

THE FREQUENCY OF OCCURENCE OF SUPERNUMERARY SPERM IN RAT OVA ¹

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SIX FIGURES

INTRODUCTION

The presence of intact spermatozoa within the perivitelline spaces of ova of various mammals has been reported from time to time. Many of the observations have been limited to fixed and sectioned material used in the study of the fertilization process (Sobotta and Burckhard, '11, in the rat; Lams and Doorme, '08, in the mouse; Lams, '13, in the guinea pig; van der Stricht, '10, in the bat; van der Stricht, '11, and Hill and Tribe, '24, in the cat). Although accessory sperm have been reported in the ova of some mammals studied in the fresh condition (Gilchrist and Pincus, '32, in the rat; Lewis and Wright, '35, in the mouse; Pincus and Enzmann, '32, in the rabbit; Heape, 1886 in the mole), no efforts were made to determine the frequency of occurrence or fate of these sperm. It is generally agreed for mammals that although more than one sperm may penetrate the zona pellucida only one enters the oöplasm to effect fertilization. On the other hand, the penetration of more than one sperm into the oöplasm itself is a common phenomenon in the eggs of birds, reptiles, urodeles, selachians and insects (Frankhauser and Moore, '41). Polyspermy has likewise been reported in

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the eggs of *Amphioxus* (van der Stricht, 1896), *Platynereis* (Just, '15), and in the echinoderms, *Toxopneustes variegatus* and *Asterias forbesii* (Wilson and Mathews, 1895).

The term "polyspermy" has been used to mean either the penetration of more than one sperm into the oöplasm, with the subsequent development of more than one sperm nucleus, or the presence of one or more sperm in the perivitelline space of a fertilized ovum. Since, in the literature dealing with fertilization phenomena in the lower vertebrates and invertebrates, the term "polyspermy" has been employed more widely to signify the penetration of the oöplasm by more than one sperm we suggest that either the term "super-numerary sperm" or "accessory sperm" be utilized to denote the presence of non-fertilizing spermatozoa in the perivitelline space.

During the course of an investigation on the total number of spermatozoa reaching various segments of the reproductive tract in the rat (Blandau and Odor, '49) our attention was drawn to the frequent appearance of intact supernumerary sperm within the perivitelline spaces. The present report is concerned with a study of the frequency with which supernumerary sperm occur in rat ova.

MATERIALS AND METHODS

Data for this study were obtained from 46 mature female albino rats of Wistar and Vanderbilt strains. The onset of sexual receptivity was used as a reference point for estimating the time of ovulation. It has been shown that ovulation is completed in most animals approximately 10 hours after the onset of heat (Boling, Blandau, Soderwall and Young, '41). Females in heat were placed with two vigorous males and allowed to remain overnight. If on the following morning the vaginal smears showed the presence of sperm the animals were killed by decapitation at the intervals indicated in table 1.

The reproductive tract was immediately removed and the cornua separated from the oviducts by cutting the utero-

TABLE 1
The frequency and number of supernumerary sperm found in ova of rats killed approximately 2 to 100 hours after ovulation

NO. OF ANIMALS	HOURS AFTER OVULATION ANIMALS KILLED	O V A		ACCESSORY SPERM							
		Number recovered	Number fertilized	Number with accessory sperm		Number of ova with 1	Number of ova with 2	Number of ova with 3	Number of ova with 4	Number of ova with 5	
				Ova	%						Ova
14	2-8	148 ¹	103 ²	70	18	18	12	5 ³	1		
7	10-14	66 ³	61	92	11	18	7	3	1		
14	25-53	140 ⁴	135 ⁵	96	38	28	26 ⁵	6	3 ⁵	2 ⁵	1
8	74-100	69 ⁶	68	99	18	26	12	6			
Total		423	366	87	85	23	57 (67%)	20 (24%)	5 (6%)	2 (2%)	1 (1%)

¹ Includes one empty zona pellucida.
² Includes one fragmenting ovum with two intact sperm in perivitelline space.
³ Includes three fragmenting ova.
⁴ Includes one ovum not described and one empty zona pellucida.
⁵ Includes: one single-cell ovum with three intact sperm, but no fertilizing sperm; one fragmenting ovum with one intact sperm; and one fragmenting ovum with 4 intact sperm.
⁶ Includes one empty zona pellucida.

tubal junctions. Under a binocular dissecting microscope the encapsulated ovary was cut away. A fine pipette filled with Ringer's solution was inserted into the fimbriated end of the oviduct and held in place by a fine forceps. Ringer's solution was repeatedly forced through the tube to make certain that all ova had been flushed out. If, at the time of killing, the ova had reached the cornua they were similarly washed out. Any granulosa cells surrounding the ova were removed by suspending them in a 0.1% solution of the enzyme hyaluronidase.² The ova were transferred to clean cover slips by means of a fine pipette. The cover slip was then sealed with vaseline to a hanging-drop slide and the ova immediately examined under the high power of a microscope.

OBSERVATIONS

The frequency of occurrence and number of supernumerary sperm found in ova of rats killed two to 100 hours after ovulation is summarized in table 1.

Of the 148 ova recovered between two to 8 hours after ovulation, 103 (70%) were fertilized. From one to three accessory spermatozoa were present in 18% of the fertilized ova, all of which were in the one cell stage and surrounded by a large number of granulosa cells which had to be removed by the addition of the enzyme hyaluronidase.

The 66 ova obtained between 10 and 14 hours after ovulation were still in the one cell stage and, except in a few instances, were free of granulosa cells. Of these 18% had one to three sperm in their perivitelline spaces. The ovum in figure 1 was obtained from an animal killed during this interval but not included in the table. Five spermatozoa were found within its perivitelline space.

Twenty-eight percent of the 140 ova recovered from animals killed between 25 and 53 hours after ovulation contained supernumerary sperm. A majority of the ova recovered during this period were in the two cell stage (figs.

² The enzyme hyaluronidase was supplied by the Ortho Research Foundation, Raritan, New Jersey, through the courtesy of Dr. Carl G. Hartman.

3 and 4), although some of them were still in the one cell stage (fig. 2). This group contained two fragmenting ova with accessory sperm and a single ovum with three intact sperm within the perivitelline space, but with no evidence of a fertilizing sperm.

In the period between 74 and 100 hours after ovulation 26% of the 68 fertilized ova contained accessory sperm. Twelve of the ova were in the morula stage (fig. 5), and the remainder were blastocysts lying free within the cornua (fig. 6).

It is of interest that although the heads of the supernumerary sperm were frequently detached from the neck pieces they retained their original contour and appearance even in blastocysts. In every instance the entire accessory sperm had completely passed through the zona pellucida and was lying within the perivitelline space. This latter fact was repeatedly confirmed by transferring ova from the hanging drops to microscopic slides where they were gently flattened with a cover slip and observed under an oil immersion lens.

Of the 85 ova in which accessory sperm were observed 67% contained only one sperm; 24% contained two; 6% contained three; 2% contained 4 and one contained 5 (table 1). In none of the fertilized ova containing accessory spermatozoa was there evidence that more than one sperm penetrated the oöplasm.

The cornua of three animals were thoroughly flushed out on the 5th day after ovulation or at a time when the blastocysts were no longer confined within their zonae pellucidae. Two normal appearing sperm heads were found adhering to the periphery of one of 9 blastocysts recovered from one of these animals. No evidence of other sperm or parts of sperm was found after careful search of the washings.

DISCUSSION

There are two major factors contributing to the dearth of information concerning the presence of supernumerary spermatozoa within the perivitelline spaces of mammalian

ova. First, most of the investigators studying the processes of fertilization have used fixed and sectioned ova in which the perivitelline space is obliterated, so that identification of accessory spermatozoa is very difficult. Second, it has been only recently that the enzyme hyaluronidase has been made available, the use of which permits the removal of granulosa cells and greatly simplifies observations on early fertilized ova.

The environment within the perivitelline space of ova is such that even though the heads of sperm occasionally become detached, they nevertheless may retain their normal configuration up until the time of implantation. On the other hand, many free sperm in the oviducts and cornua have begun to undergo disintegration as early as 24 hours after insemination (Blandau and Odor, '49). At the time of implantation, when the zona pellucida is lost, the accessory sperm are cast into the lumen where they probably disintegrate.

Supernumerary sperm have been observed from time to time in other species such as the rabbit (Pincus and Enzmann, '32) and the cat (Hill and Tribe, '24). In the mouse, two to three accessory sperm may enter the perivitelline space as early as two hours after ovulation (Lams and Doorme, '08; Lewis and Wright, '35).

The presence of supernumerary sperm has been recorded by Lams ('13) in the two- and 4-cell stages of guinea pig ova. He maintains that they have no significance since they remain extraovular where they degenerate and disappear. Heape (1886) observed that accessory sperm occur frequently in the mole where as many as 20 may be found within a single ovum.

Our observations indicate that supernumerary sperm have no effect on the developmental rate of the ovum. It is interesting that in several instances fragmenting ova were found which contained a number of sperm in the perivitelline spaces. It is likely that the oöplasms of these ova were already defective before ovulation. The number of ova containing ac-

cessory sperm probably depends upon the number and vitality of the sperm reaching the ampulla within certain specific intervals after insemination (Blandau and Odor, '49).

SUMMARY

A study has been made of the frequency with which supernumerary spermatozoa appear in the ova of rats.

1. Of a total of 423 ova recovered from 43 rats between two and 100 hours after ovulation 366 were fertilized; 85 of these, or 23% contained one to 5 supernumerary sperm.

2. Of the 85 ova containing accessory sperm 67% had one; 24%, two; 6%, three; 2%, 4 and 1%, 5.

3. In all fertilized ova containing accessory sperm only one spermatozoon had penetrated the oöplasm.

4. Except for a separation of the head from the neck piece the accessory sperm showed no evidence of disintegration in any of the developmental stages including the late blastocyst.

5. The presence of supernumerary sperm has no effect on the rate of development of the ovum.

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PLATE 1

EXPLANATION OF FIGURES

All ova photographed in a hanging drop of Ringer's solution. $\times 450$.

1 Ovum recovered approximately 11 hours after ovulation. Five sperm are present within the perivitelline space.

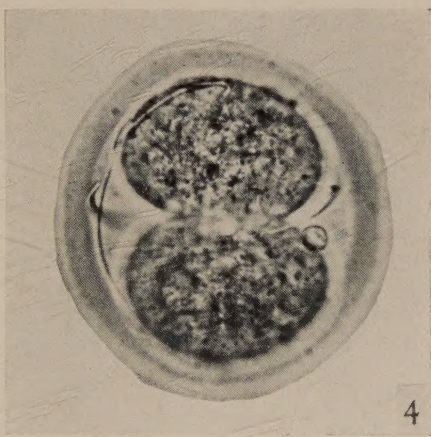
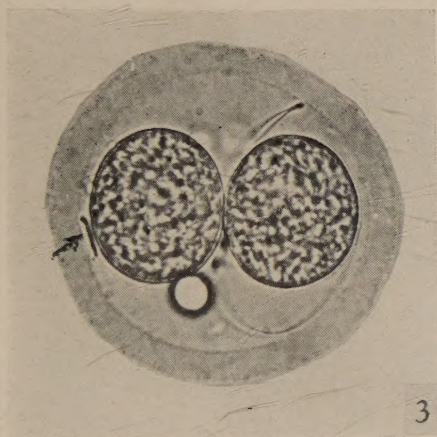
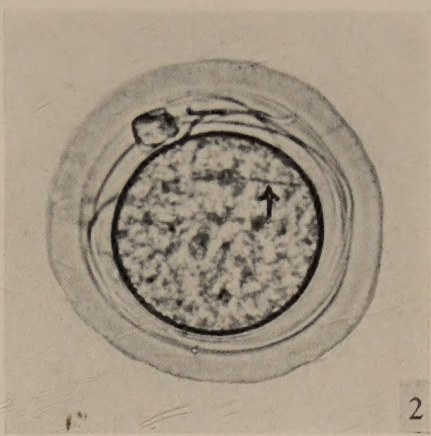
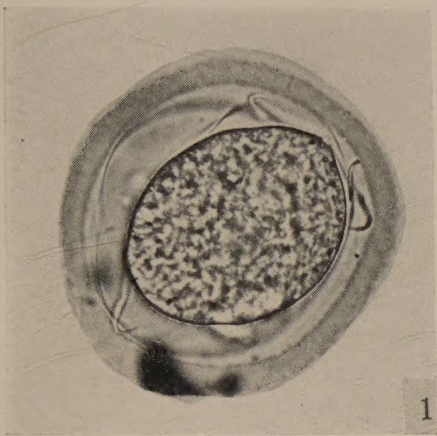
2 Ovum recovered approximately 26 hours after ovulation. Five sperm are present in the perivitelline space. Arrow points to the fertilizing sperm in the oöplasm.

3 Two-cell ovum recovered approximately 52 hours after ovulation. One accessory sperm present with head detached. Arrow points to location of head.

4 Two-cell ovum recovered approximately 51 hours after ovulation. Three accessory sperm are present, two with the heads still attached to the neck piece and one with its head detached.

5 Eight-cell stage recovered approximately 74 hours after ovulation. Arrows point to single accessory sperm. The head is detached but of normal appearance. This ovum had been supravitaly stained with janus green.

6 Blastocyst recovered approximately 100 hours after ovulation. Two accessory sperm with heads detached but of normal contour were found within the perivitelline space.



A SIMPLIFIED MACHINE FOR MAKING GROUND SECTIONS OF TEETH OR BONE

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TWO FIGURES

The machine described simplifies the arduous task of making tooth or bone sections. Not only is a greater degree of accuracy made possible but the time required is greatly reduced. Two revolving discs are used which double the speed of the grinding process. The specimen is mounted on one disc which rotates counterclockwise to another disc which is faced with an India stone. This machine is in use in our laboratories.

DESCRIPTION OF THE MACHINE

Figures 1 and 2 show the general appearance and certain details of the machine. The figures are largely self-explanatory but certain parts will be described.

The discs (no. 29 and no. 30) are $6\frac{1}{4}$ " in diameter and are made of brass. Three are used, two for grinding and one for the attachment of the specimen. Fine and medium grit India stones, 6" in diameter, are used for grinding and are cemented into a recess cut in disc no. 30. The plain disc no. 29 has shallow concentric rings cut into its face so that the specimens may be more securely cemented to this surface. However, the apparatus is more useful when brass pans are used for the attachment of the tooth or bone material. Each pan has an inside diameter of $1\frac{1}{4} \times 1 \times \frac{5}{32}$ " and is attached to the disc by a single bolt. Several holes for bolts are drilled in the plain disc, each a different distance from the center. This allows for changing the pan and assures a more

uniform wearing of the India stones. The pans are filled with artificial stone or plaster of Paris and after drying, are made perfectly smooth by dressing with the fine India stone. The specimen to be ground is cemented to the artificial stone or plaster and actually is more adherent to these materials than

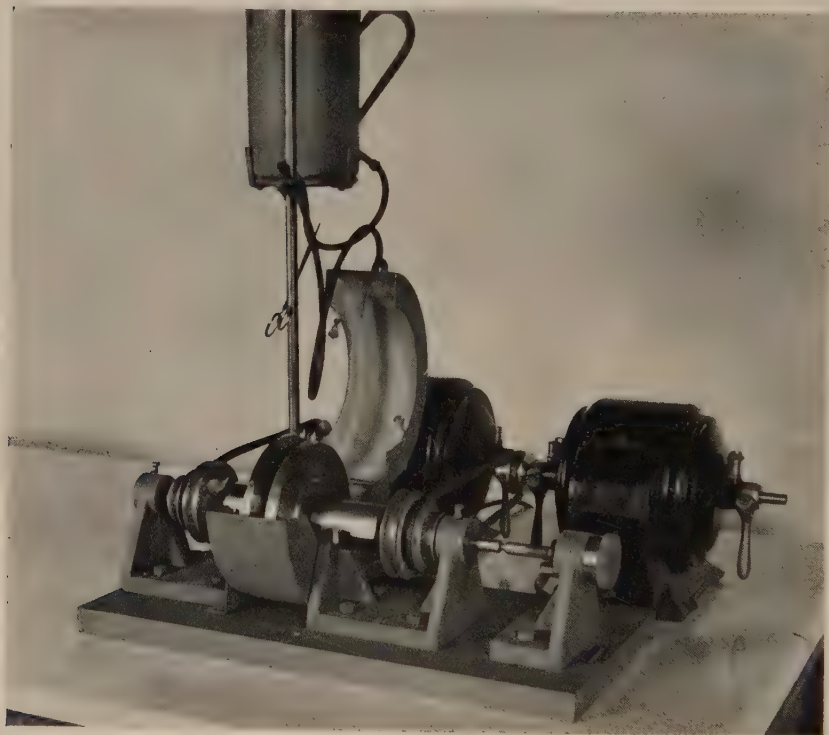


Figure 1

when cemented directly to the disc. This method of attachment facilitates the grinding process in that the pans may be interchanged and time is not lost while waiting for the cement to dry or while the specimen is being dissolved from the pan.

The plain disc (no. 29) and the grinding discs (no. 30) are threaded so that either can be placed on shafts (no. 4 or no. 5). Shafts no. 4 and no. 5 are mounted on the machine in such

a way that the centers are offset $\frac{3}{4}$ " which reduces the chance of a groove being worn into the stone. If the offset is more than 1" and the specimen is mounted near the periphery of the disc, the specimen while revolving will not make constant contact with the stone.

The micrometer shaft no. 17 makes contact with shaft no. 5 by a case hardened spindle. Spring no. 12 holds shaft no. 5

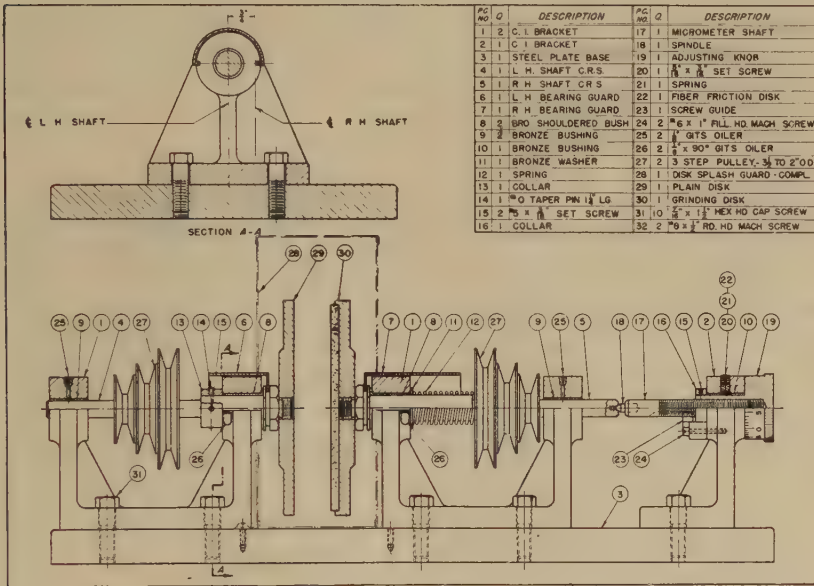


Figure 2

against the spindle. A screw guide no. 23 fits into shaft no. 17 and prevents the shaft from revolving but allows movement in a linear direction. The fiber disc no. 22 holds the micrometer in adjustment. The adjusting knob of the micrometer no. 19 is divided into 50 spaces, and when it is turned one space the shaft moves $1/1000$ ".

Other important features of the machine are the bearing guards no. 6 and no. 7, which reduce the amount of water and grit which fall into the bearings. Section A-A shows how the bearing guards fit into position. While the machine is in

operation the discs (no. 19 and no. 20) are enclosed by pan and cover guards. The cover guard is hinged to the back portion of the pan and swings forward over the discs. The specimen is kept wet during the grinding operation by a stream of water directed between the discs by a size 16 hypodermic needle. The hypodermic needle is inserted into an orthodonic brass tube which extends through and is soldered to the cover guard. Water is supplied from a small container supported by a ring stand rod which is attached to the base and extends above the machine. Water is drained from the pan guard by a small tube which is connected to an aspirator pump.

DESCRIPTION OF TECHNIQUE

The tooth and bone material are fixed and stored in a 10% formol solution. Suitable sections of bone, approximately $1\frac{1}{2}$ mm in thickness, are cut from the shaft of a long bone. The sections are rubbed on a piece of fine sandpaper to smooth the surfaces. Preparation of teeth is more difficult because of the problem of securely holding the teeth while the desired sections are ground or cut. Either a fine saw or a medium stone attached to a lathe motor is used in preparing the rough sections. At this stage the preparation should be about $1\frac{1}{2}$ or 2 mm in thickness and it is generally desirable to have one plane of the section near the center of the tooth. Both surfaces are smoothed on fine sandpaper.

The section of bone or tooth is cemented, with a thin layer of Duco Household Cement, to disc no. 29 or preferably on the artificial stone of one of the brass pans. The cement is allowed to dry thoroughly and if the latter method of attachment is used, the pan is bolted to the disc. Because of the variation in the thickness of the sections, only one section can be ground at one time. If the specimen is mounted so that the smoothest surface is on the outside, it may be possible to finish this surface by using only the fine grit India stone. The surface must be ground until it is perfectly flat. The preparation is then removed by dissolving the cement with acetone. A hand micrometer is used to measure the

thickness of the preparation and the specimen is cemented again for grinding with the unfinished surface exposed. At this time, the stone is brought into contact with the specimen by adjusting the micrometer knob (no. 19). This contact should be strong enough so that the motors cannot turn the discs. The adjusting knob on the micrometer is now turned back until the discs begin to revolve and a reading on the micrometer adjusting knob is made. Since the thickness of the specimen is known, its thickness can be ascertained during any stage of the grinding process. The medium grit stone is used for grinding the preparation to almost the desired thickness. The fine grinding stone is then inserted into the machine, but since the stones are not the same thickness, it is necessary to determine the thickness of the preparation again by using the method described above. The grinding and polishing process is then completed with the fine stone. The preparation is removed from the disc or pan with acetone, allowed to dry or transferred to xylol and mounted in thick balsam.

ACKNOWLEDGMENT

This device in its initial form was designed by Mr. J. G. Sentinella, machinist for the Department of Physics of the State University of Iowa, in consultation with Dr. C. L. Drain, then Head of Pedodontics in the College of Dentistry. Later certain modifications were incorporated by Drs. J. D. Boyd and James Stewart. The present machine is similar except that a more efficient micrometer has been designed, bearing guards have been added, and separate removable pans for the attachment of specimens have been used.

INTRAVITAL STAINING OF BLOOD VESSELS BY ACID DIAZO DYES¹

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FOUR FIGURES

INTRODUCTION

Acid diazo dyes are abundantly deposited as microscopic granules in the cytoplasm of macrophages and cells which have to do with the excretion of such materials. Many injured or necrotic cells are diffusely stained by the same dyes (Williams and Hodge, '43; Williams, '47, '48a, '48b). A few apparently normal tissue elements are also diffusely stained by them. Among these are mural components of blood vessels (Petroff, '22; Glasunow, '26; Okuneff, '26; Cappell, '29 a and b; Duff, '32; Anitschkow, '33; Williams, '47; Williams and Frantz, '48). The reports of Cappell, Duff, and of Anitschkow furnish a review of most of the studies of vital staining of blood vessels.

The purpose of this report is to describe the reactions of blood vessels of mice and rats to small amounts of subcutaneously injected acid azo dyes.

MATERIALS AND METHODS

Mice weighing 20 to 30 gm received subcutaneous injections of 0.1 or 0.2 cm³ of 0.5% aqueous or saline solutions of trypan blue (color index 477) or chlorazol fast pink (c.i. 353) daily for three to 12 days. Animals were killed 4 to 48 hours subsequent to the cessation of dye injection. One hundred of

¹ Aided by grants from the Medical Research Fund of the Graduate School of the University of Minnesota.

the mice received no treatment other than the dye. An equal number were from experiments which included treatment with chlorinated hydrocarbons or cardiac glucosides (Williams, '47 and 48a). Some of the mice were pregnant and others were treated with dye during the first week following parturition, some of which were nursing their litters during this period. Another group of mice received similar amounts of dye every 4th day during a 12 day period. Ten rats were untreated except for daily injections for 4 to 10 days of 0.4 to 1 cm³ of 0.5 or 1% solutions of one of the dyes mentioned above. In addition 10 rats were also treated with digoxin (Williams, '47), 10 were fed a restricted (low protein) diet, and 10 received toxic amounts of chlorazol fast pink but no trypan blue (Williams and Hodge, '43). Tissues were fixed and prepared for study by methods previously reported (Williams and Frantz, '48). The fixatives used were a modified Lavdowsky fluid (Williams and Frantz, '48), acetone, acetone followed by absolute alcohol (Emmel, '46), and a mixture of acetone and absolute alcohol in equal parts. Paraffin was used for embedding and sections were cut at 10 to 15 μ .

OBSERVATIONS

General. The best examples of vital staining of mural components of blood vessels were seen in material fixed by one of the three acetone methods. Similar staining was observed in tissues fixed in aqueous reagents but the amount of dye remaining in the vessels seemed less in some cases. Blood vessels were studied in all tissues except bone. Veins were not vitally stained. The staining of arterial vessels was not consistently specific or uniform except as described in detail subsequently. The sites of greatest and most regular staining were the elastic elements of large arteries and the intimal and medial areas of small arteries, between and including the internal and external elastic laminae. Such elastic laminae were deeply and precisely stained while the intervening medial area was lightly and diffusely colored or unstained. These small arteries were relatively lightly

stained in such organs as the liver and spleen although the abundant intramacrophagic deposition of these azo dyes proves the circulation of the dye through these viscera. The present report is limited to normal vessels but it should be mentioned that edema alters the vital staining of pulmonary elements including the vessels of the lung. Among small arteries those of the kidney showed the greatest amount of staining. This organ is the major excretion site of these dyes and some of the tubular epithelial cells contain large amounts of stain. Similar vessels and some of the capillary endothelium in gravid and post parturient uteri were fairly deeply stained.

Acute treatment with chlorinated hydrocarbons and cardiac glucosides had no effects upon the staining of the blood vessel walls. Large toxic amounts of chlorazol fast pink had no significant effects upon the blood vessels. The injury produced by these agents in the myocardium and in the parenchyma of other viscera has previously been described (Williams, '47 and '48a; Williams and Hodge, '43; Tarail and Williams, '48).

Small arteries of kidney. The internal and external elastic laminae of these renal vessels were uniformly stained as if the elastic fibres had selectively bound the dye (figs. 1-3). As mentioned before, acetone "fixation" methods seemed to accomplish the greatest retention of dye in the small arteries and use of these methods resulted in fixation of the blood vessels to a degree far superior to that of adjacent parenchyma. The interval subsequent to the terminal injection of dye (4 to 48 hours) did not seem directly related to the amount of residual staining of the elastica. Very slight mural staining occurred in the arterial walls of mice receiving only three injections of dye during a 12 day period and which were killed 48 hours later.

In mice injected daily with dye for 5 to 10 days the vital staining of the walls of the small renal arteries was as follows. No dye was observed in the intima. The internal elastic lamina was deeply stained and the pattern of staining

followed the contour of the elastic fibres. The subjacent media was usually very lightly tinted. The next layer, the external elastic lamina, was stained in the same fashion as the internal one, but usually slightly less so. The larger renal arteries were not routinely stained and the same was true for arterioles and capillaries.

In rats receiving non-toxic amounts of these azo dyes the mural staining of blood vessels was similar to that just described for mice. Following toxic amounts of chlorazol fast pink the site of greatest tissue injury and necrosis was renal and the vessels showed considerable mural staining. The vital staining of elastic laminae was not proportionally increased. Very large doses of azo dyes produce a great amount of atypical vital staining (Williams and Hodge, '43).

Aorta and heart. Grossly, the aorta was stained. Only a sparse diffuse staining of the aortic media was observed in Lavdowsky-fixed specimens of cardiac portions of aortae from 20 mice which had been treated with dyes for less than a week. The elastic fibres were not well outlined by the intravital staining. The elastic medial fibres of acetone-fixed thoracic and abdominal aortae of mice treated with trypan blue for 10 days were heavily stained by the vital dye (fig. 4). There was heavy staining of elastic mural elements of Lavdowsky-fixed cardiac portions of "great" arterial vessels in rats which had received trypan blue for only 4 days (1 cm³ of a 1% solution, subcutaneously). In aortae of mice and rats the staining was almost entirely limited to the media. Fibrous elements other than elastic were not colored.

The endocardium, myocardium and epicardium of normal hearts showed no vital staining although macrophages contained dye. Staining of coronary arteries was less than that of renal arteries.

Livers. The abundant deposition of diazo dyes in sinusoidal and other hepatic macrophages demonstrates the intravascular passage of large amounts of dye through the organ. Vital staining of small arteries in the livers of mice and rats was

usually limited to a diffuse medial coloration plus a slightly greater staining of the elasticae. The precise delineation of mural elastic elements common to the kidney was not attained in the liver.

Gravid and post parturient uterus. Many uterine elements, particularly those in decidual and implantation sites, stored the dyes. Macrophages were numerous and some of the dye seemed interstitial and extracellular. In quantity the amount of mural staining of small arteries was less than in the kidney and some aortae but in general greater than in other organs. The mediae showed fairly heavy diffuse staining but the elasticae were not as well demarcated by vital staining as were those of renal vessels. The best examples of endothelial staining were seen in endometrial capillaries.

Other organs and tissues. Diffuse medial staining similar to that observed in some of the intrahepatic arteries occurred in the small arteries of other viscera and tissues. In the results reported here the staining was not sufficient to warrant further discussion. Studies of the central nervous system were quite limited and insufficient material was fixed by acetone methods. Internal elastic laminae of small cerebral arteries were faintly stained in rats receiving large amounts of trypan blue.

Local staining. At injection sites diffuse staining of almost all tissue elements was observed. Such reactions do not correctly pertain to the phenomenon of true vital staining since they represent a local response to a relatively enormous amount of concentrated dye stuff.

Elastic tissue in general. The dyes showed no affinity for elastic tissue except that of the arterial walls. Elastic fibres within the viscera showed no sign of vital staining. Also, the majority of elastic fibres in the adventitia and the perivascular connective tissue were not stained. Macrophages in the latter area often contained granules of dye and the quantity of dye in these cells was not related to the amount of mural staining present in the adjacent blood vessel.

DISCUSSION

When small, non-toxic amounts (0.1 to 0.2 cm³ in 25 gm mice) of diazo dyes are injected subcutaneously or intraperitoneally some part of the injection mass is absorbed by vascular or lymphatic capillaries and therefore directly or indirectly reaches the venous circulation, the cardiac and pulmonary circuits, and then the arterial circulation which transports the dye to sites of deposition and excretion. The latter rather rapidly eliminate a greater part of the absorbed dye. Also, the fairly large amount remaining at the injection site must be deducted. The dilution of dye by the volume of normally circulating and local intercellular fluid must also be considered. The acid azo dyes are almost entirely excreted by the kidney.

Therefore it is obvious that only a relatively small amount of dye is available for the staining of or absorption and storage by non-excretory cells or tissues. In the non-toxic amounts used here these dyes are normally deposited in renal epithelia (which excrete them), in phagocytic cells and in certain portions of the walls of blood vessels. In addition some fat cells show deposition of azo dyes (Bremer, '38; Chang, '40; Williams and Hodge, '43). As a result of injury many cells are atypically stained by these dyes (Williams, '47, 48a, 48b).

In the present study there was no evidence of arterial damage although some of the animals were subjected to the effects of toxic agents (Williams, '47 and '48a). In such animals the responses of the arterial wall to the dye was the same as in rats and mice which had received no treatment other than injection of dye. As early as 1922 it was reported that the walls of some blood vessels could be stained by essentially local application of diazo dyes and by intravenous injection or perfusion of relatively large quantities (Petroff, '22; Glasunow, '26; Okuneff, '26). Some of these findings were related to injury of the vascular wall (Duff, '32; Anitschkow, '33). These early studies showed that trypan blue stains mural vascular elements. It is difficult to compare

these earlier results with the present study since formerly the major interest was in the larger arteries, mostly the aorta. There seems little doubt, however, that the fundamental phenomenon is the same. Cappell ('29a and '29b) has discussed the staining of the intima and internal elastic lamina. He and the earlier workers concluded that staining represents direct absorption for the vascular lumen and via the intima. The significance of the validity of such an interpretation seems overshadowed by the potential importance of the almost specific staining (binding?) of the elastic fibres. The amount of staining in these sites seemed dependent upon the concentration of elastic fibrils. 'When they occurred as a lamina or bundles the staining was intense. When the fibrils were more diffusely arranged the vital staining was less obvious. It seems that local structural factors or metabolic conditions were responsible for the staining of the elastic tissue. The staining of elastic fibres in these areas was specific. Elastic fibres in other areas and in viscera in general were not vitally stained by these dyes. Such fibres were present in perivascular areas but were not stained. Granules of dye within adjacent macrophages showed that the dye had reached such sites.

The determining factor in vital staining is not local availability of the dye but responses of the tissues to the dye. Apparently, most normal cells show no visible reaction to these dyes in that the dye either does not enter the cell or is never present in demonstrable quantities. The best known reactions to the azo dyes are phagocytosis by macrophages and the absorption and excretion mechanisms of epithelial cells of the renal proximal convoluted tubules. The elastic membranes of certain vessels are one of the few normal elements which are clearly stained by these dyes.

Duff ('32) presented an excellent review of studies of vital staining of blood vessels and an extensive investigation of such staining in the rabbit's aorta subsequent to intravenous injection of trypan blue. He described considerable patchiness of staining and also variations in amount of

staining following comparable quantities of dye in the arch of the aorta. The thoracic aorta was more uniformly stained. In most instances staining was greater in adventitia and outer media which was interpreted as indicating diffusion from the more heavily vascularized peripheral mural areas. In two instances relatively heavy staining was observed in inner medial sites and the author correlated such with the location of minute nutrient vessels entering the media from the lumen. The patterns of staining observed in the present study are not in complete agreement with those of Duff. In mice and rats the mural vital staining of large blood vessels was concentrated in the media and more specifically almost limited to elastic elements. The whole medial layer seemed evenly stained as to inner and outer areas. The adventitia was unstained although macrophages in perivascular connective tissue contained dye. However, the experimental conditions as well as the species differed from those in Duff's studies.

King ('38) injected enormous amounts of dye (2.5 to 15 mg in 1 or 2% solutions) intravenously into 17 to 19 gm mice studied the results in 30 μ sections. Diffuse staining of numerous elements including blood vessel walls was reported. It seems likely that the dose of dye was within the toxic range and further, the volume of intravenously injected fluid caused hydremia and the resulting vital staining would not be valid.

Local staining of elastic and collagenous fibres at sites of injection of these dyes has long been recognized and may precede intramacrophagic deposition of the dyes. Essentially all elements in such sites are similarly stained (see Downey, '16 and '17).

Further study is required before attempting to correlate vital staining of uterine vessels with the structural and functional changes characteristic of the gravid and post parturient states of that organ. Most studies of uterine vital staining have been limited to macrophages or to the possible transmission of dyes from maternal to foetal tissues

(Wislocki, '20; Nicol, '35; Hamilton, '39). A multitude of circumstances and alterations may account for the relatively high degree of vital staining. Among these would be the hypertrophic and proliferative vascular changes related to implantation and gestation, and the regressive processes subsequent to parturition. The reactions to dyes may be reflections of the local hemodynamic processes which oxygenate and drain the gravid uterus. Hyperemia, stasis, local intravascular pressure and gaseous tensions may be responsible for the increased vital staining. The superiority of endothelial staining in the endometrium may be a result of the above vascular factors. The occurrence of vital staining of capillary endothelium at injection sites (of dye) represents a different circumstance since in such areas the endothelial cells are actually deluged by a relatively tremendous amount of dye and therefore it is observed intracellularly because of its quantity. Further, so much dye in such a very high local concentration may damage the endothelium and result in an injury or pre necrosis type of vital staining. The endothelial cells are the first membrane for transfer of dye to its definitive place of deposition. In relation to interval of passage the amount of dye stuff may not be sufficient to be observed within such cells by the usual methods now available. The same is probably true during the pre segregation absorptive or diffusion processes of macrophages (Williams and Hodge, '43). Downey ('16 and '17) observed that in injection sites the endothelium of capillaries was well stained during the early stages of dye absorption.

An obvious limitation in these studies has been the destaining effects of reagents used in the preparation of tissues for study. The acetone methods of fixation-dehydration seems to give some improvement on this score. No satisfactory explanation of the greater staining of mural elements of small intrarenal arteries seems to exist. Some aspect of the histotechnical procedures may be responsible but it is not apparent. The renal excretion of these dyes does not spe-

cifically involve a greater concentration of them in the arterial system of the kidney. There are no known special characteristics of the elasticae of small renal arteries which make them different from splanchnic vessels of similar size. No accepted concept of normal intrarenal blood flow would seem readily to account for increased absorption or diffusion of these dyes in relation to the blood vessel wall even if such activities are responsible for the increased staining. In relation to the aorta the high local level of circulating dye and the abundance of elastic fibres may explain the striking staining of that vessel.

SUMMARY

Mice and rats were given subcutaneous injections of either trypan blue or chlorazol fast pink. Tissues were fixed in acetone with or without the addition of absolute alcohol, or in a modified Lavdowsky's fluid. Deparaffinized sections were studied without counterstaining, or after counterstaining with acetone solutions of contrasting dyes, the purpose being to avoid subjecting the sections to aqueous or alcoholic reagents.

Portions of arterial walls were stained intravitaly by these diazo dyes. The greatest amount of mural vascular vital staining was observed in the abundant elastic fibres of the great arteries and in the elastic laminae of small arteries. There was no generalized staining of elastic fibres in other sites. Acetone methods of fixation seemed to aid in the preservation of vital dye in the small arteries but were of less significance in this respect in the large ones.

Animals thus treated with dye included some subjected to dietary (protein) restriction, or treatment with chlorinated hydrocarbons or cardiac glycosides. These relatively acute experimental procedures produced myocardial, hepatic and renal lesions, but did not significantly alter the vital staining of mural elements of blood vessels.

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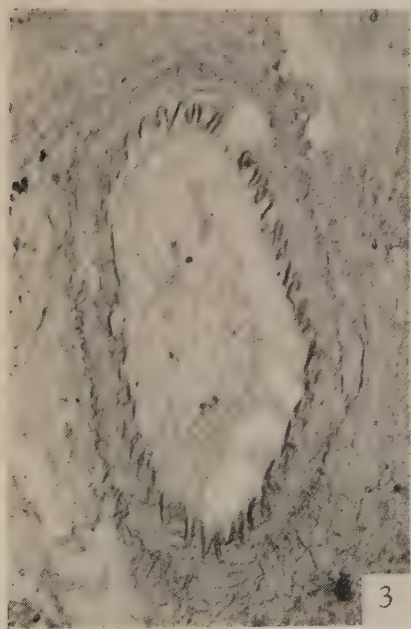
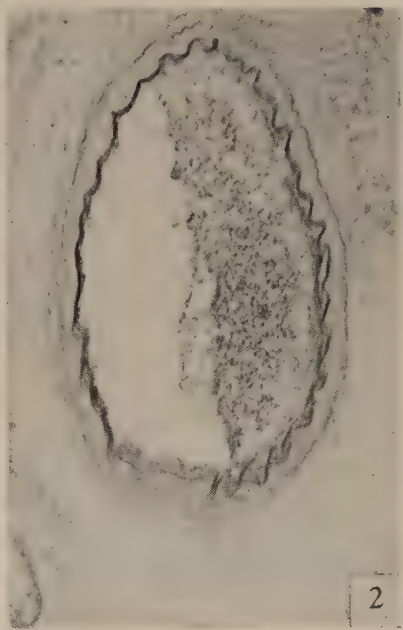
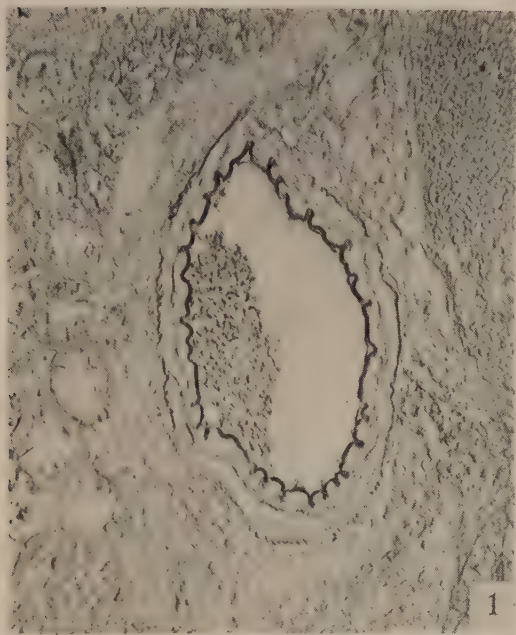
PLATE 1

EXPLANATION OF FIGURES

Note: The sections are from acetone-fixed material and were not counter-stained. All staining shown is intravital. Photographed with carbon arc illumination, green filter and polychrome plate.

1, 2, and 3 Small arteries of mouse kidneys. Figures 1 and 2 show staining of elastic membranes by intravital chlorazol fast pink. Figure 3 is from a mouse treated with trypan blue and the small dark particles are perivascular macrophages containing the dye. The dose of dye in this mouse was insufficient to cause any considerable deposition within tubular epithelium. $\times 200$.

4 Portion of abdominal aorta of mouse treated with trypan blue. Lumen of vessel is at the right. Note heavy staining of medial elastic elements. Other mural elements were unstained. Periadventital macrophages contained dye. $\times 750$.



THE ANATOMY OF THE DORSAL APONEUROSIS OF THE HUMAN FINGER AND ITS FUNCTIONAL SIGNIFICANCE

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SIX FIGURES

Studies of the anatomy and function of the dorsal aponeurosis of the finger have been infrequent since the work of Duchenne (1885). New interest in this subject by anatomists has arisen recently, however, because modern reconstructive surgery requires a more comprehensive knowledge of this part of the hand. Duchenne, in his "Physiologie der Bewegungen" (1885) states that the dorsal aponeurosis which is formed by the tendons of the extensor communis, interossei and lumbricales muscles, provides a mechanism for coördination of the movements of the second and third phalanges. From electro-physiologic experiments, as well as from the study of a hand in which the fingers were moved by artificial tendons and strings, he concluded that normal finger movements could be obtained only when the tendons of the extensor and interossei muscles were intact. Duchenne discussed the work prior to his time. The subject has been brought up to date in a review of the literature by Sunderland ('45). According to Sunderland "it is misleading to speak of isolated, individual and specific actions of the extensor digitorum, lumbricales and interossei muscles. They function as well integrated and coördinated groups in every movement of the digits . . ." The present work indicates that the dorsal aponeurosis gives the morphological basis for the integration and coördination of the extensor and interossei muscles.

The most detailed account of the dorsal aponeurosis is found in Poirier's Human Anatomy ('04) and agrees in general with our own. We were not able, however, to confirm his statement that the extensor tendon is inserted into the base of the proximal phalanx and we agree with Kaplan ('45) who states that he was able to find only a short fibrous slip, which was never tendinous, running from the palmar surface of the extensor tendon to the base of the proximal phalanx, and this only in 38.5% of 140 fingers examined.

Montant and Baumann ('38) and Baumann and Patry ('43) showed by micro-dissection that the interossei and extensor communis tendons intermingle their fibers to form ultimately the median and lateral tendons of the dorsal aponeurosis. On the dorsal side of the second phalanx, a triangular lamina of connective tissue, joining the two lateral tendons with each other prevents both tendons from gliding off the base of the second phalanx. The authors agreed with Hauck ('22), who described the volar shift of the lateral bands during flexion of the proximal interphalangeal joint. Bunnell ('44) also confirmed this finding: "On flexion of these joints, however, the lateral forks must short cut across the middle joint, so that the above limited excursion of the main lateral band will allow both the middle and distal joints to be flexed at the same time." In Bunnell's opinion a fascial sheet, extending from the lateral band to the collateral ligament and to the base of the middle phalanx, causes this volar shift. Benninghof ('39) also observes that the lateral band passes along the lateral side of the first interphalangeal joint. He states, moreover, that the tendon crossing dorsal to the joint opposes flexion more strongly than would a tendon passing alongside the joint. Undoubtedly Benninghof noticed this important feature of the dorsal aponeurosis, but he gave a static description only and overlooked the general concept of its significance.

The purpose of the present work is to give an anatomical description of the dorsal aponeurosis, and exposition of the normal patterns of movement of the finger and a correlation of the morphological features with the functional phenomena.

ANATOMY

The dorsal aponeurosis is easily exposed by removing the loose subcutaneous connective tissue. The extensor communis tendon on the dorsal surface of the first phalanx is flanked on both sides by the tendons of the interossei and lumbricalis muscles which together form the lateral wings of the dorsal

ABBREVIATIONS FOR ALL FIGURES

E.C., extensor communis tendon
 F.A., lateral communis band
 I.T., interosseus tendon
 L.B.T., lumbricalis tendon
 L.T., lateral tendon
 M.I.B., medial interosseus band
 M.T., middle tendon

O.B., oblique band of retinacular ligament
 R.L., retinacular ligament
 S.I.L., superficial intertendinous lamina
 T.B., transverse band of reticular ligament
 T.T., terminal tendon

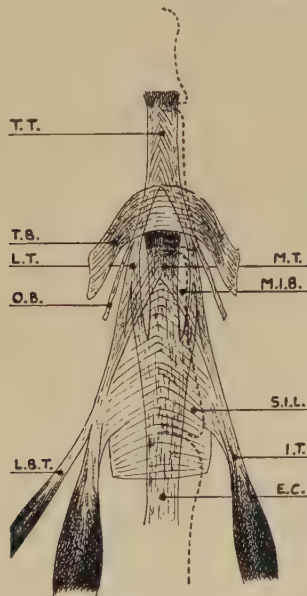


Fig. 1 Dorsal aponeurosis represented schematically in dorsal view. The two components of the retinacular ligament, viz., the oblique band (O.B.) and the transverse band (T.B.) have been detached from their proximal insertion and have been spread out laterally so as to bring them into view. At the right side the interrupted line outlines the metacarpal bone and the phalanges. Notice the position of the lateral tendon (L.T.), lateral to the trochlea of the first phalanx.

aponeurosis (fig. 1). The appearance of wings is produced by a thin lamina of connective tissue (*lamina intertendinea*) which arises from the interosseus tendon and extends dorsally toward the communis tendon. It consists of two layers, superficial and deep; the superficial one (*lamina intertendinea superficialis*) passes over the communis tendon, the deep one is firmly adherent to the lateral border of the communis tendon.

At the distal end of the first phalanx, the relation between the tendons is changed because the extensor communis tendon by fanning out separates into three parts, whereas the interosseus tendon sends a strand of fibers in a spiral course over the diverging communis fibers. As a result, the fibrous layer joining the communis and interosseus tendons is no longer discernible. The lateral band of the communis tendon (fig. 1, 2 F.A.) intermingles with the lateral or palmar portion of the interosseus tendon to form the lateral tendon (*fasciculus lateralis* or Poirier's *langnette latérale*) (fig. 1, 2 L.T.) which continues distally to the third phalanx. The remaining median portion of the extensor communis tendon is joined by the medial band of the interosseus tendon and the superficial intertendinous lamina to form a very dense, strong bundle of fibers which adheres to the capsule of the proximal interphalangeal joint and with which it is attached to the proximal border of the second phalanx. This is the middle tendon (*fasciculus medius* or Poirier's *langnette moyenne*) (fig. 1, 2 M.T.).

The dorsal aponeurosis at the level of the first interphalangeal joint, therefore, is composed of a middle and two lateral tendons. The middle tendon terminates by attaching to the proximal border of the second phalanx, so that the further continuation of the dorsal aponeurosis is formed by the lateral tendons only. The latter cross the proximal edge of the second phalanx, lateral to the insertion of the middle tendon, and on the dorsal surface of this phalanx converge until they ultimately join to form the terminal tendon which inserts into the base of the third phalanx.

An important portion of the terminal tendon of the dorsal aponeurosis which does not originate in the lateral tendons has not hitherto been fully recognized. This structure, the retinacular ligament (fig. 2, R.L.), is composed of two parts, a transverse layer which is spread over both lateral tendons (fig. 1, T.B.) and a very slender but strong band which merges with the most lateral fibers of the terminal tendon (fig. 1, O.B.). The first or broad thin ligament, recognized by Bau-

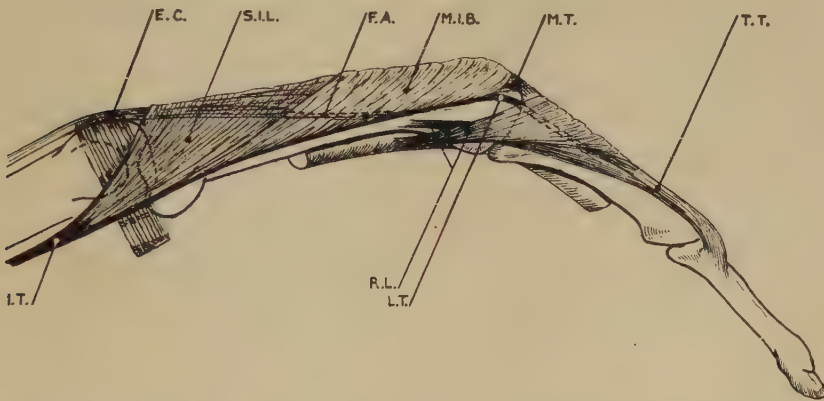


Fig. 2 Lateral view of the dorsal aponeurosis with the retinacular ligament. Notice that the lateral band of the extensor tendon contributes to the lateral tendon (broken lines, F.A.), while the interosseus tendon contributes its medial band to the middle tendon. The retinacular ligament, composed of (1) the transverse band and (2) the oblique band of the terminal tendon, attaches to the first phalanx as well as to the ventral tendon sheath.

mann and Patry and by Bunnell passes across the proximal interphalangeal joint. Some of its fibers reach as far as the tendon sheath of the ventral surface of the first phalanx to which they adhere. The fibers of the second or oblique ligament pass underneath the transverse part of the retinaculum and insert into the lateral border of the first phalanx (fig. 2). In some cases fibers of the oblique band pass along the lateral surface of the first phalanx towards its base. The lateral aspect of the proximal interphalangeal joint is crossed, therefore, by two ligaments, (a) the lateral tendon and (b) the

retinacular ligament. At the level of the proximal interphalangeal joint, the dorsal aponeurosis as a whole, forms a hood which is drawn over the joint, further reinforcing it at the sides.

In order to determine the relation of the lateral tendon and the retinacular ligament, especially its oblique part, to the axis of movement of the proximal interphalangeal joint preliminary measurements were made according to the "Polbahnen"-construction of Fick ('10). This method is based on the principle that when a bone is moving in one plane from one position to another, we are able to construct an axis around which this bone might be supposed to have turned to get from the initial position to the second one. In a following paper we will give a report of a more detailed X-ray analysis based on the same method. It was found that the lateral tendon passes dorsal and the oblique band closely ventral to the axis. The relation of the lateral tendon and oblique band to the axis is not constant, however, because both are displaced ventrally when the joint is flexed.

PATTERNS OF MOVEMENT

I. Functional aspects of the flexor digitorum profundus

Although the deep flexor is inserted on the terminal phalanx, it causes the series of phalanges to flex. With few exceptions, it is impossible, by active muscular effort, to flex the distal interphalangeal joint by itself and passive flexion of the distal phalanx also is followed by flexion of the proximal interphalangeal joint, unless prevented by external force. Moreover, although the "neutral" position of the finger is in partial flexion the proximal interphalangeal joint cannot be maintained in this neutral position when the finger is flexed by the profundus. The reason for this is that the shifting of the dorsal aponeurosis along the second phalanx, produced by flexion of the distal phalanx, causes flexion of the proximal interphalangeal joint. In other words the effect of contraction

of the flexor profundus is to a large extent determined by the arrangement of tendinous elements in the dorsal aponeurosis.

II. Functional aspects of the flexor digitorum sublimus

Flexion of a particular finger by the superficial flexor muscle alone can be attained only by keeping the other fingers extended. As soon as the proximal interphalangeal joint is flexed, hypertension of the distal phalanx, normally present in the fully extended finger, can no longer be maintained. The action of the extensor diminishes as flexion in the proximal interphalangeal joint increases so that the profundus may increase the flexion of the third phalanx without interference from the extensor. If the second phalanx is flexed at least 80° without contracting the profundus, the third phalanx is fully released from both flexion and extension and we observe the phenomenon of the loosened third phalanx (fig. 6). The position of the third phalanx, however, is not strictly dependent on the position of the second phalanx because in any given position of the second phalanx it is possible to flex the third phalanx further to some degree against resistance.

III. Functional aspects of extension

Extension of the terminal phalanx is possible only if accompanied by extension of the second phalanx and similarly extension of the second phalanx invariably results in extension of the terminal phalanx. A rather constant feature of the extensor pattern, therefore, is that the finger must be unrolled in both interphalangeal joints simultaneously. Extension of the second phalanx, moreover, is hindered by a flexed position of the third phalanx and if the latter is fully flexed, passive extension only of the second phalanx is possible and only with considerable and often painful outside force. Under such forced extension, a decrease of a few degrees in the flexion of the third phalanx is almost always observed, indicating that flexion of the third phalanx is able

to lock the proximal interphalangeal joint to a certain extent.

THE FUNCTION OF THE DORSAL APONEUROSIS IN RELATION TO ITS STRUCTURE

The dorsal aponeurosis may be divided into the following components, represented schematically in figure 3 in an extended position, (a) the middle tendon, (b) the lateral tendons, (c) the lateral bands which split off from the communis tendon to join the lateral tendons, (d) the medial bands which arise from the interosseus tendons and join the middle tendon, and (e) the retinacular ligament.

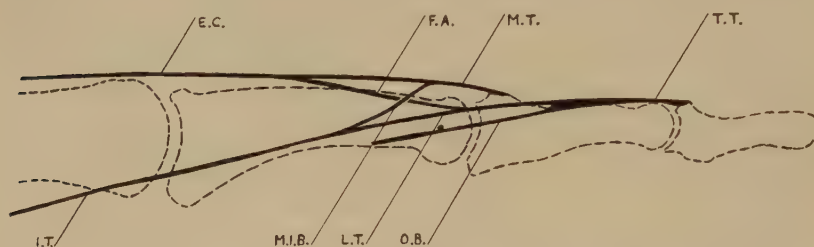


Fig. 3 Schematic drawing of the dorsal aponeurosis. The finger in extended position.

I. Forces active during flexion of the third phalanx

When the third phalanx is flexed, either by the action of the deep flexor or passively, the terminal tendon shifts distalward along the second phalanx. This pulls on the lateral tendons which, by means of the lateral communis bands, pull the communis tendon distalward, slackening the middle tendon. In this way the most powerful extensor influence on the proximal joint is inactivated, and only the lateral tendons, which have no proper pulley, remain to exert their extending influence. At the same time, the distal movement of the terminal tendon tends to increase the tension on the retinacular ligament, especially on its oblique part (fig. 3-5, O.B.) and since the latter is situated ventral to the axis of rotation of the proximal interphalangeal joint, it will tend to flex this joint.

In figure 4 the distal phalanx has been flexed, causing the terminal tendon to shift approximately 3 mm distalward. The lateral communis bands have caused the middle tendon to shift distally the same distance. If the second phalanx is now

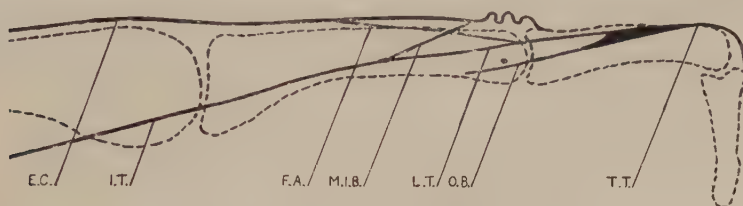


Fig. 4 Terminal phalanx flexed. Notice this is a forced flexion, the second phalanx can only be kept in the extended position by external pressure. The dorsal aponeurosis is shifted distally about 3 mm. Tolerance in the middle tendon approximately 3 mm. Strong tension on the retinacular ligament.

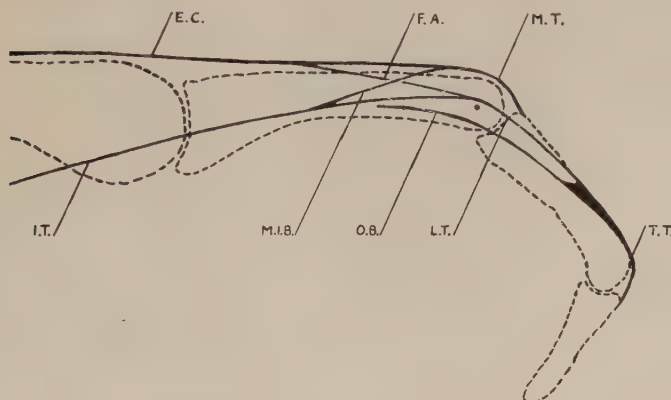


Fig. 5 The second phalanx has given way to the action of the retinacular ligament and eventually that of the deep flexor. Notice that the lateral tendon has left its position on the dorsal surface of the trochlea and has shifted distally about 0.7 mm. All further flexion of the second phalanx will release the third phalanx through the medium of the medial interosseus band.

flexed by means of the retinacular ligament (fig. 5), the middle tendon will again be taut after 60° – 70° of flexion. During this movement the lateral tendons, running alongside the trochlea of the proximal phalanx, only shift approximately 0.7 mm. Obviously these lateral tendons exert their extending

influence on the second phalanx in a much less effective manner than a tendon running over the trochlea, and the greater the flexion the more unfavorable is their position for extension. In figure 5 the lateral tendons run immediately dorsal to the axis of rotation of the joint, their extending force diminished but their stabilizing influence increased. Under these circumstances the tension of the taut retinacular ligament, tend-



Fig. 6 The second phalanges flexed beyond 90° to free the third phalanges. The dotted lines show the range of free motion of the terminal phalanges. Since the third phalanges cannot be extended as long as the second phalanges keep this position, the nails of the two middle fingers cannot be made to touch each other.

ing to flex, is more than a match for the extending force of the lateral tendons which have no pulley. This tautening of the retinacular ligament together with the simultaneous slackening of the middle tendon may be studied favorably in dissecting room material.

II. Forces active during flexion of the second phalanx

Flexion of the second phalanx immediately puts tension on the middle tendon which, by means of the medial interosseus

bands, is transferred to the lateral tendons also. Since the medial interosseus bands eventually run over the trochlea of the proximal phalanx and the lateral tendons run beside the trochlea, greater displacement of the interosseus bands causes the lateral tendons to become less tense. Also, by decreasing the distance from the axis of rotation of the joint, the distal displacement of the lateral tendons becomes greater than that required for the existing amount of flexion of the proximal interphalangeal joint. In this way a certain amount of laxity of the terminal tendon is provided, which allows the third phalanx to be flexed by the profundus without opposition from the extensor. It will now be evident that extreme flexion of the second phalanx allows the the third phalanx to escape entirely from the action of the extensor tendon. At 90° of flexion of the second phalanx, the lateral tendons are drawn distalward by the medial interosseus bands to such an extent, that they can no longer exert any influence on the third phalanx.

III. Forces active during extension

The extensor communis extends the finger in the metacarpophalangeal and interphalangeal joints, for the extensor not only exerts its action via the middle tendon but, through the medium of its lateral band, also via the lateral tendon. The lateral tendon is displaced proximally over a greater distance than that required for the extension of the second phalanx, so that the third phalanx is extended together with the second phalanx.

Extension of the second phalanx with the third phalanx partly flexed would have to be executed mainly by the lateral tendons. Apart from the fact that this would seem improbable, such a movement is entirely prevented by the retinacular ligament, which has been made taut. Thus flexion of the third phalanx implies a corresponding flexion of the second phalanx.

Extension at the proximal interphalangeal joint also implies a corresponding extension of the distal joint. The retin-

acular ligament, which passes ventral to the axis of the joint, is put under tension and exerts a force on the terminal tendon, resulting in extension of the third phalanx.

Certain quantitative data connected with figure 3 should be stressed. With full flexion of the third phalanx, the middle tendon slackens sufficiently to allow 60° – 70° of flexion in the proximal interphalangeal joint. With further flexion of the second phalanx both the middle and lateral tendons are shifted distalward 2 mm according to our measurements. The latter tendons thereby lose their last power over the third phalanx. The lateral tendons, distal to the medial interosseus bands, are now fully relaxed and cannot be tautened, either (a), by tension from a proximal direction exerted by the extensor and interossei because their action ends at the proximal edge of the second phalanx or (b), by action from a distal direction, because the third phalanx is already fully flexed.

The ventral shifting of the lateral tendons during flexion of the proximal interphalangeal joint may be explained further. The lateral tendons, inclined to take the shortest route between the proximal edge of the second phalanx (their bony hypomochlion) and the interosseus tendons (their origin), will shift ventrally because the proximal edge of the second phalanx is displaced ventralwards. Although the lateral tendons may, by this process, shift ventral to the axis of rotation, no effective flexing force results because neither the third phalanx, nor the interossei and extensor muscles will act on these tendons, the forces of the latter coming to a dead end on the proximal edge of the second phalanx. It is evident that at the moment the third phalanx falls again under the influence of the lateral tendons, this phalanx may in turn tauten the lateral tendons. This may happen at 60° – 70° of flexion of the second phalanx, and our experiments show that in this degree of flexion the lateral tendons are again situated dorsal to the axis of rotation of the joint. It follows that no locking of the proximal interphalangeal joint through the medium of the lateral tendons is ever possible.

SUMMARY

1. A part of the dorsal aponeurosis hitherto partially described in literature has been named the retinacular ligament. It consists of two parts: (1) a transverse band spread out over the lateral tendons as they converge on the dorsum of the terminal phalanx, and (2) an oblique part, composed of the most lateral fibers of the terminal tendon of the dorsal aponeurosis.

2. The retinacular ligament passes ventral to the axis of rotation of the first interphalangeal joint and is attached to the first phalanx, some fibers radiating into the palmar tendon sheath.

3. During flexion of the third phalanx this ligament is tautened and causes a simultaneous flexion of the second phalanx.

4. This simultaneous flexion of the proximal interphalangeal joint may be reinforced by the flexor digitorum profundus, and is greatly facilitated by the slackening of the middle tendon of the dorsal aponeurosis.

5. During flexion of the proximal interphalangeal joint the lateral tendons pass alongside the trochlea of the first phalanx which results in a decrease of their extending influence.

6. Flexion of the terminal phalanx locks the first interphalangeal joint in a certain degree of flexion by a tautening of the retinacular ligament.

7. An attempt has been made to correlate the patterns of movement of the different digital muscles with the anatomical features of the dorsal aponeurosis.

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VACUOLATION AND THE RELEASE OF HEPARIN IN MAST CELLS CULTIVATED IN VITRO¹

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SIXTEEN FIGURES

INTRODUCTION

Recently mast cell rich tissue from a mast cell tumor of a dog (Bloom, '42) was found to contain an anticoagulant of a potency so great that in the case of one tumor each unit weight yielded upon extraction "almost 50 times as much heparin as has been obtained from the richest normal source of the most active form of heparin" (Oliver, Bloom, and Mangieri, '47). The extraction of this substance in such massive amounts confirmed the contention of Holmgren and Wilander ('37) and others (Jorpes, '46) that the mast cell produces heparin.

Throughout this work an intriguing fact was apparent, the blood clotting time of the dogs was normal even in animals which were host to tumor tissue measurable in pounds.

Since mast cells produce heparin, the presence of a normal clotting time in tumor bearing dogs might mean one of two things; first, that heparin was not liberated from the cells in appreciable quantity or second, that having passed out of the cells it was destroyed or inactivated.

From the work of Paff, Bloom, and Reilly ('47) the conclusion seems justified that the mast cells in these tumor bearing dogs did not release heparin in appreciable quantity.

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This conclusion is based on the fact that heparin being a sulfonated polysaccharide reacts metachromatically with toluidine blue and no evidence was seen in tissue culture that indicated passage of a metachromatic substance out of the cell and into the surrounding medium.

The question which arose in our minds was how do mast cells release their secretion? What morphological changes occur in the process? We had seen no evidence of heparin discharge in several hundred toluidine blue stained mast cell cultures which were maintained in prime condition. After trying various procedures it was found that, by the simple method of permitting cultures of the tumor to age, some of the cells underwent changes which are suggestive of secretory activity and which throw light upon controverted observations made by previous workers in normal mast cells.

CULTIVATION OF THE TUMOR

The technique employed in cultivating the mast cells was essentially that described in previous papers by Paff, Bloom, and Reilly ('47) and Paff, Montagna, and Bloom ('47). Small fragments of a dog mast cell tumor were planted in roller tubes lined with a film of chicken blood plasma. One or two drops of chick embryo extract were added to facilitate clotting. When a firm clot had formed $2\frac{1}{2}$ cm³ of dog serum and $\frac{1}{2}$ cm³ of chick embryo extract were added and at intervals of one to two days the tubes were examined under the microscope. Fragments which had grown well were removed and planted in hanging drops consisting of a trace of embryonic extract, a drop of dog serum and a drop of chicken blood plasma. For a period of about one month the fragments were transplanted as soon as degenerative changes appeared. At the end of this time we began our studies by deliberately permitting some of the cultures to age. Instead of transplanting the fragments every three, 4 or 5 days we extended the period for as long as 7, 8, or 9 days. Photographic records were kept of many of the cells and metachromasia was studied by placing two or three drops of a sky blue Tyrode-toluidine

blue solution on the surface of the clot and watching the staining reaction under the microscope. This procedure usually consumed from one to three hours of time.

OBSERVATIONS ON AGEING CULTURES

The first cells which grew out from the fragments planted in hanging drops were either irregularly shaped cells with long tenuous processes, oftentimes no wider than a granule (figs. 1 and 2), or epithelioid cells growing in the form of sheets (fig. 4). Both types were sometimes found in the same culture and all of the cells were rich in granules (fig. 3).

As the cultures aged, vacuoles appeared in some of both the irregularly shaped cells with processes and in the epithelioid cells. Since the pattern varied three types of activity will be described.

1. *Vacuolation and granule discharge (Clasmatosis)*

In the first type vacuoles arose as a prelude to a form of clasmatosis. A few cytoplasmic vacuoles appeared in a mast cell and increased markedly in size (figs. 5, 6, and 7). Rupture of these large vacuoles was accompanied by the discharge of granules from the cell into the surrounding medium. The vacuoles themselves contained no granules, but their rupture apparently so disorganized the cell's plasma membrane as to permit granules in the vicinity of the vacuoles to leave the mast cell (fig. 8).

2. *Vacuolation and granule dissolution*

In the second type, vacuoles appeared in the cytoplasm not in small numbers but in amounts which eventually reduced the cell to a discrete mass of foam (figs. 9 and 11). In this type of activity the granules contributed to the formation of the vacuoles and the vacuoles did not attain the relatively large size of those described above in cells which had undergone clasmatosis. When the cell attained its foam-like appearance only a few granules remained. These lay in the

cytoplasm between the vacuoles (fig. 11). With continued degeneration of these cells vacuoles passed off into the media and were absorbed. This form of dissolution of the cells can be hastened by agitating the area in which they lie.

3. *Vacuolation in epithelioid cells*

The third type was found in mast cells growing in the form of epithelioid sheets (fig. 4). The cells became rounded and their shape and appearance at this time were highly suggestive of developing granulocytes (fig. 13). Vacuoles appeared primarily along the periphery of the cells and sometimes attained a size as large as the main mass of the cell (figs. 13, 14, and 15). Originally the vacuoles were spherical (figs. 14 and 15) but the development of new ones and an increase in size of those already present, caused them to assume the appearance of being compressed (fig. 16).

DISCHARGE OF METACHROMATIC MATERIAL

When young cultures were stained with a very dilute Tyrode-toluidine blue solution most but not all of the cells showed metachromatic granules in abundance. As the cultures aged the number of cells whose granules showed metachromasia decreased somewhat. The reason for this is to be found partly at least in the fact that metachromatic material was observed to leave the cells not only as granules in the process of clasmatosis but also in solution as will be seen below. In figure 10, a cell is shown in which some of the granules are discrete and intensely metachromatic while others are vague and splotchy. These last are discharging metachromatic material into the ground substance of the cytoplasm and some of this material is present in two of the three vacuoles found in the cell. Most important is the fact that a cloud of the same metachromatic material was detected passing from the cell into the surrounding medium. This phenomenon was observed in only a few of the hundreds of cells visible in a culture but it was unmistakable and was checked by independent observers. Such discharging cells

were usually surrounded by many cells showing metachromatic granules but nothing more.

The unstained cell in figure 9 was later stained and the vacuoles were observed to be faintly but distinctly metachromatic. Some of the granules were discrete and deeply stained, others were only lightly stained. No discharge of material was seen. Cells of this type may take on the appearance seen in figure 11. This cell was stained and metachromatic material was present not only within the vacuoles but diffusely between them and when first observed an evanescent halo of metachromatic material was seen outside of the cell. In considering this form of cell, as was noted above, the vacuoles can become dissociated from the cell with continued ageing of the culture. This adds a third means of discharging metachromatic material from the cell and gives us vacuoles in addition to granules and solution.

Figure 12 is a type of stained cell rarely seen in our cultures. It contains one very large strongly metachromatic vacuole and several smaller ones. The large vacuole occupies a position suggestive of the granular free area noted in previously studied tumors (Paff, Bloom, and Reilly, '47). The granules in this cell stained faintly or not at all and many had undergone disintegration.

DISCUSSION

The answer to the question as to what morphological changes occur in the process of the physiological liberation of heparin from the mast cell *in vivo* need not necessarily be found in observations of the above type but certainly they must be taken into account as a possibility. Although the changes described are essentially degenerative it is remarkable that for practically every observation we have made on the tumor mast cell *in vitro* a counterpart can be found in the literature of the normal cell *in situ* (Michels, '38).

The first thing which became obvious was that when grown in tissue culture and observed each day tumor mast cells are quite active. They divide mitotically and probably amitoti-

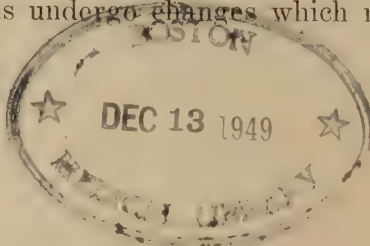
cally; they are remarkably polymorphous in form, existing as either cells with long processes or rounded or ovoid cells; they undergo ameboid movement (Paff, Bloom and Reilly, '47); they secrete a substance which responds metachromatically to toluidine blue and which expresses itself in the form of granules of varying staining intensity (Oliver, Bloom and Mangieri, '47, and Paff, Bloom and Reilly, '47) or as a diffusible solute traceable from the cell to the surrounding medium, or as a liquid confined to vacuoles which themselves may rupture or float into the surrounding medium.

The fact that metachromatic material was observed to leave the cell interested us most. Since this substance is undoubtedly heparin or some form of it we thought that perhaps we could further verify its presence by simple clotting tests. Our results were negative but we believe this was due to the fact that the material was present in too small amount since, as observed above, relatively few cells in a single culture show it free in the medium when stained with toluidine blue. If one were fortunate enough to obtain a culture in which many of the cells were in the liberation phase of secretory activity then a clotting test would doubtless reveal the presence of the anticoagulant.

Finally our work implies that the secretory cycle of heparin production and liberation involves degenerative changes and even death of the mast cell. This removes the cell from the descriptive limits of the text book but only a little reflection will reveal the desirability of this. Dogmatism in defining a mast cell and too ready use of the term "artifact" in relationship to such phenomena as granule dissolution or formation of vacuoles have contributed ideas which are too circumscribed in the light of the dynamic physiological role which the mast cell plays in producing heparin.

SUMMARY AND CONCLUSIONS

When tissue cultures of tumor mast cells of dogs are permitted to age the cells undergo changes which result in the



liberation of a metachromatic substance which is presumably heparin or some form of it.

In some cells a few large vacuoles form and then rupture with the result that granules are scattered into the surrounding medium.

In other cells granules undergo solution and metachromatic material may be discharged directly into the surrounding medium.

In still other cells granules contribute metachromatic material to the formation of many vacuoles. The cell resembles a mass of foam. The vacuoles may discharge their metachromatic substance directly to the surrounding medium or the vacuoles may actually float into it.

These findings imply that the secretory cycle of heparin production and liberation involves degenerative changes and even death of the mast cell.

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PLATE 1

EXPLANATION OF FIGURES

1 Living mast cells growing from the periphery of a fragment removed from a dog mast cell tumor. Many of the cells resemble fibroblasts in shape. Note the marked granularity of the cytoplasm. Cultured for 36 days unstained. $\times 200$.

2 Living mast cells showing tenuous processes which are only one granule in width. Such processes can be traced for long distances into the medium. Cultured for 42 days. Unstained. $\times 440$.

3 Living mast cell flattened against the coverslip. Note the cytoplasmic granules. The nucleus is spherical and contains a large nucleolus. To the right of the nucleus is a granular free area. Cultured for 39 days. Unstained. $\times 970$.

4 Living mast cells growing as an epithelioid sheet. Such sheets are composed of cells varying considerably in size and having no processes. The sheets are one cell thick. Unstained. $\times 440$.

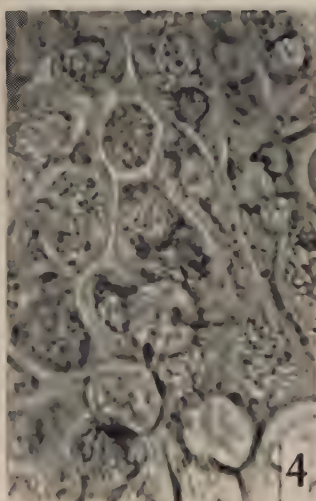
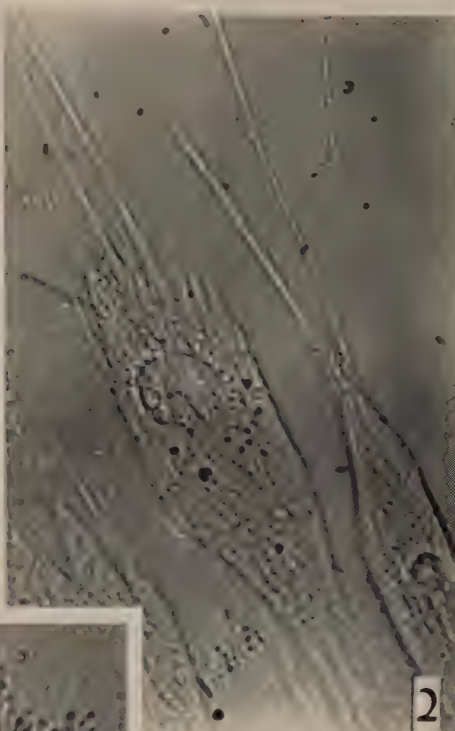


PLATE 2

EXPLANATION OF FIGURES

5 Living mast cells cultured for 41 days. Four days have elapsed since the last transplantation to fresh medium. The nucleus is ovoid and is overlaid by a few granules. Note the single discrete vacuole whose diameter is about one-third that of the cell itself. Unstained. $\times 970$.

6 Same cell as that shown in figure 5, but 6 days have elapsed since the last transplantation. The nucleus lies near the top of the photograph. Several new vacuoles have appeared. Unstained. $\times 970$.

7 Mast cell with several large vacuoles. The ovoid nucleus is near the bottom of the photograph and contains a spherical nucleolus. Seven days have elapsed since the tissue was last transplanted. This cell is not the same one that appears in figure 6, but is in the same culture. Unstained. $\times 970$.

8 Same cell as that shown in figure 7 photographed one day later. The nucleus appears to be centrally located, the vacuoles have ruptured and granules are free in the medium. Unstained. $\times 970$.

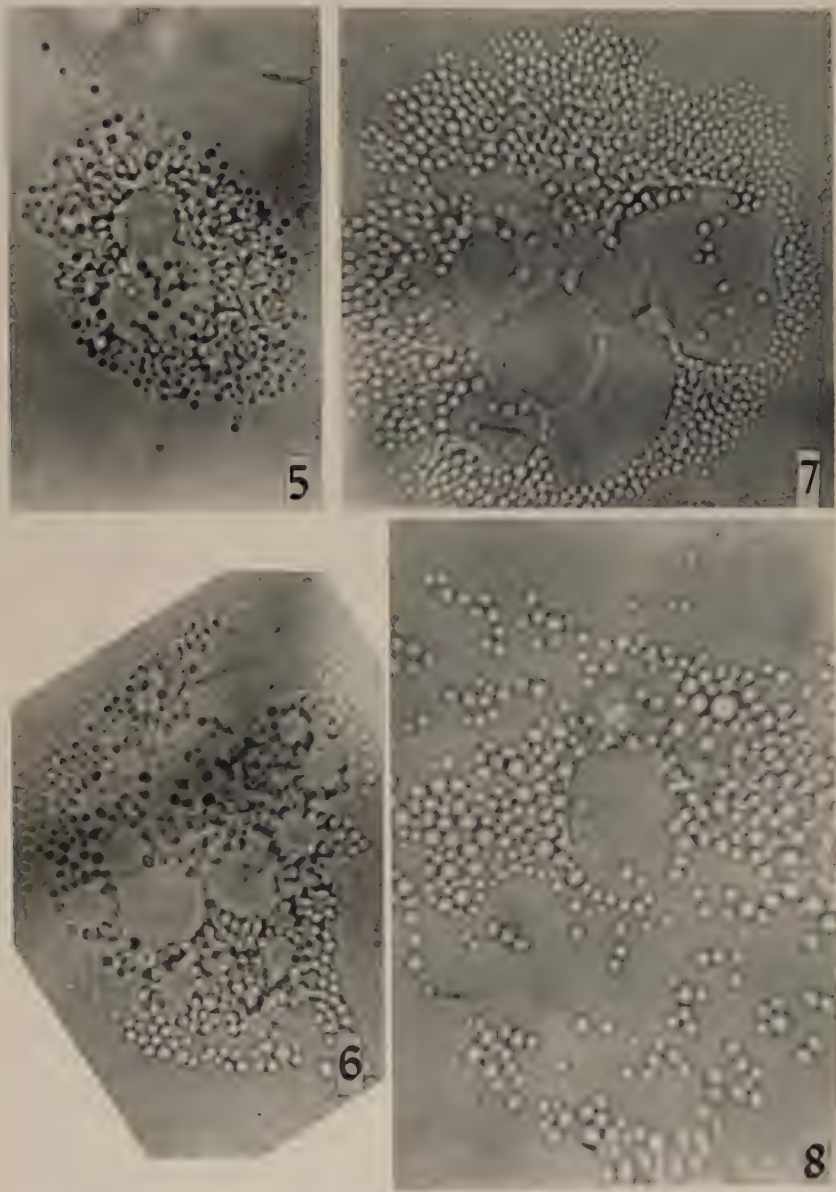


PLATE 3

EXPLANATION OF FIGURES

9 Living tumor mast cell from a culture 46 days old. Five days elapsed since the last transplantation. Note the numerous vacuoles. This cell was later stained with toluidine blue and the vacuoles and granules stained metachromatically. Unstained. $\times 970$.

10 Tumor mast cell from a 30 day culture stained with toluidine blue 7 days after the last transplantation. Although several deeply stained vacuoles can be seen the metachromatic material was being released directly to the medium by dissolution of granules. Note that the dye is not confined to the granules. $\times 970$.

11 Tumor mast cell from a 30 day culture stained with toluidine blue 7 days after the last transplantation. Degeneration in this cell had reduced it to a mass of vacuoles containing metachromatic material in varying amounts. Metachromatic material was present between the vacuoles and was passing into the surrounding medium. Note the halo surrounding the cell. $\times 970$.

12 A cell from a 28 day culture stained with toluidine blue 7 days after the last transplantation. The metachromatic material is confined for the most part to one large vacuole. This type of cell is rarely seen and was not observed to release metachromatic material. $\times 970$.

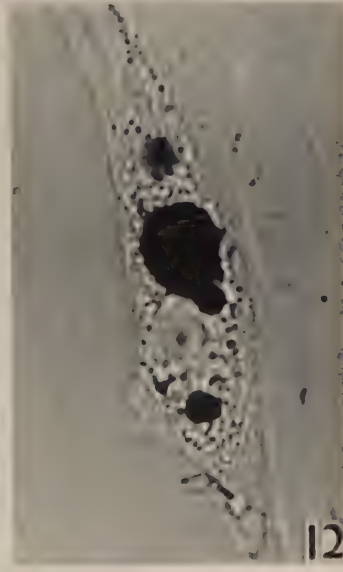
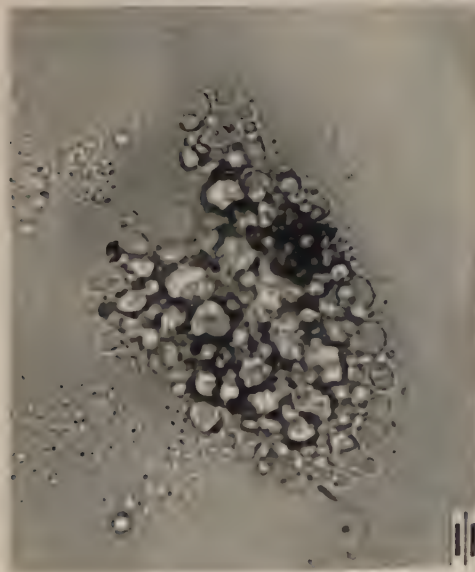
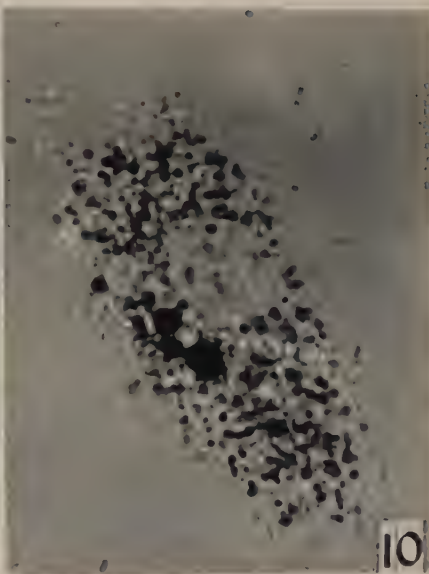
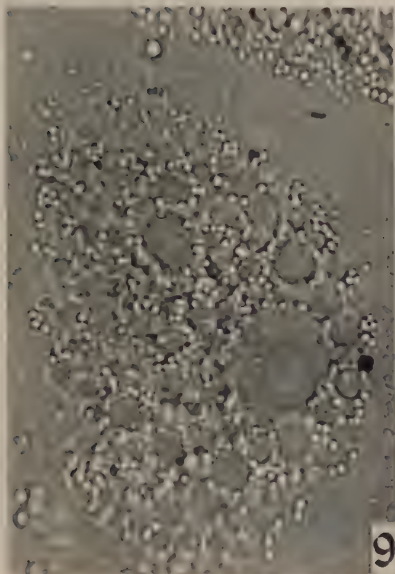


PLATE 4

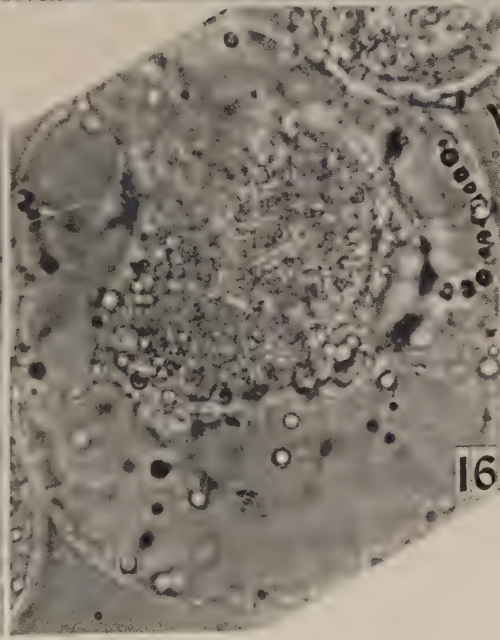
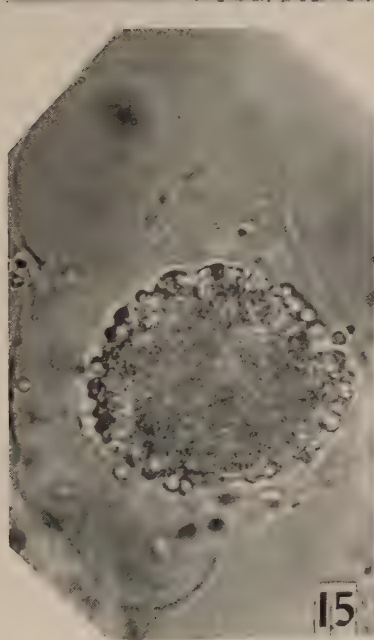
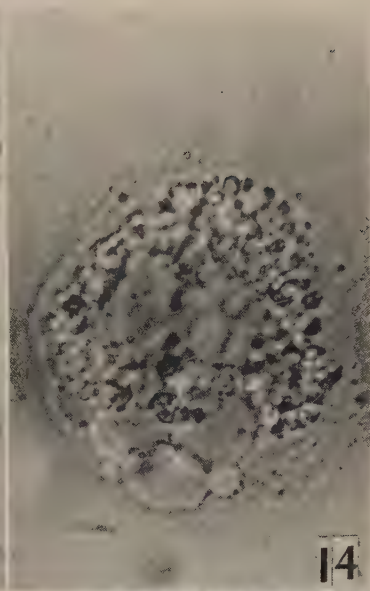
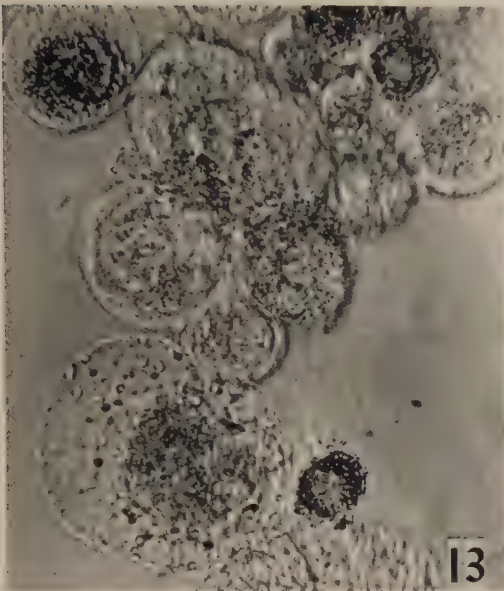
EXPLANATION OF FIGURES

13 Mast cells of an epithelioid outgrowth becoming rounded. The cells vary considerably in size due partly but not entirely to the presence of vacuoles in some of them. Culture was 33 days old and 6 days had elapsed since the last transplantation. $\times 440$.

14 Mast cell from an epithelioid outgrowth showing the early formation of vacuoles. In this type of tumor mast cell the vacuoles appear peripherally. Culture was 33 days old and 6 days had elapsed since the last transplantation. $\times 970$.

15 Mast cell in which the peripheral vacuoles have greatly increased in size but still retain their spherical shape. Such vacuoles apparently separate the plasma membrane from the main mass of the cell. This cell was later stained with toluidine blue and the large granules were intensely metachromatic but no evidence of metachromasia was found in the contents of the vacuoles. Thirty day culture; 7 days after the last transplantation. $\times 970$.

16 Mast cell in which the peripheral vacuoles are compressed into polygonal shapes probably by tension of a resistant plasma membrane. Such cells resist disintegration for long periods of time in culture. The granules are strongly metachromatic and the nucleus was identified with difficulty. Thirty-three day culture, 6 days after the last transplantation. $\times 970$.



CYTOLOGICAL DISTRIBUTION OF FAT INJECTED INTRAVENOUSLY INTO GUINEA PIGS

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EIGHT FIGURES

INTRODUCTION

These experiments were undertaken to determine the fate of intravenously administered fat as demonstrated by histological methods. One reason for interest in the problem grew out of earlier experiments by the writer on the intravenous injection of large amounts of glucose. Since the pancreas probably has a role in the control of intermediate fat metabolism and it has been demonstrated that the beta cells are altered by the administration of large amounts of glucose it was considered of interest to study the effect of the administration of large amounts of fat intravenously to determine if the hyperlipemia might have an effect on the other types of cells of the islets of Langerhans, particularly the alpha cells, as they are the type which is next in number to the beta cells.

Frazer ('46) has described the absorption of fats from the gastro-intestinal tract as including droplets of tri, di, and mono-glycerides in droplets as large as 0.3μ . Fat emulsions suitable for intravenous administration have been prepared. The preparation of such emulsions have been described by McKibben, Stare et al. ('45).

Normally there is comparatively little histologically demonstrable fat in the liver of the well-fed guinea pig. Grafflin ('40) has described the livers of a number of well-fed guinea pigs in which the amount of fat in the liver was about 2%

to 4%. The increase of fat content in the liver in starvation has been described by Mottram ('09) and Dible ('32, '34). The possible relation of increased fat in the liver to cirrhosis has been described by Connor and Chaikoff ('38), and Karsner ('43). Experimentally fatty and fibrotic livers have been produced by means of modified diets by Spellberg and Keeton ('40). While some of the most important aspects of intermediate fat metabolism, such as the rate of phosphorylation, cannot be demonstrated by the commoner cytological methods (since such studies require radioactive isotopes as in the studies of Shoenheimer, '41) still much valuable information can be obtained by ordinary histological methods.

MATERIAL AND METHODS

Fifty apparently healthy male guinea pigs weighing between 420 and 850 gm were injected intravenously with 1 or 2 cm³ of fat emulsion each containing either 15% cocoanut oil or 10% olive oil. These rates of injection amount to from 150 to 450 mg of fat per kilogram of animal weight per hour, or 3.6 to 10.8 gm of fat per kilogram of animal weight in 24 hours. The regular diet of these animals was made up of alfalfa hay, whole oats and cabbage. The animals were fed this same diet throughout these experiments, before, during, and after the injection of the fat emulsion. The emulsions were described by Stare ('45). The method of continuous intravenous injection was described in an earlier publication Woerner ('39).

The tissues to be stained for the demonstration of fat were fixed in Baker's ('44) formol-calcium solution. Frozen sections of the tissues were cut at 10 μ . Some of the tissues were embedded in gelatin before sectioning as described by Zwemer ('33). The sections were stained with hematoxylin and Oil red O and mounted in gum syrup as described by Lillie ('48). It was found that the Oil red O preparations differentiated the fat droplets better and were more suitable for photomicrography than Sudan III, or Sudan IV.

SUMMARY OF DATA ON EXPERIMENTAL ANIMALS DISCUSSED

GRAMS OF FAT IN- JECTED PER HOUR PER KILO- GRAM	GRAMS OF FAT IN 24 HOURS PER KILO- GRAM	TOTAL HOURS OF INJEC- TION	AMOUNT OF EMULSION PER HOUR	OTHER EXPERIMENTAL TREATMENT	WEIGHT OF ANIMAL IN GRAMS	SERIAL NUMBER OF ANIMAL
0.227	5.45	24	1 cm ³ of 15% cocoanut oil	none	660	60
0.148	3.55	24	1 cm ³ of 10% olive oil	24 hours rest after injection	674	69
0.694	16.66	24	2 cm ³ of 15% cocoanut oil	none	432	54
0.682	16.37	24	2 cm ³ of 15% cocoanut oil	96 hours rest after injection	440	55
0.588	14.11	68	2 cm ³ of 15% cocoanut oil	none	510	52
0.460	11.04	24	2 cm ³ of 15% cocoanut oil	250 mg choline chloride each 12 hours dur- ing and 24 hours after injection	652	58
none	none	none	none	96 hours of inanition	640	72

OBSERVATIONS

Of the 50 animals which were injected the following were chosen for cytological description because the tissues received adequate prompt fixation and because they appear to be characteristic of the stages they represent.

Liver

A normal control or stock animal, well-fed on the standard diet used for all the experimental animals of this series. Animal 59, figure 1.

Most of the hepatic cells of the sections of this animal's liver contain no fat demonstrable with Oil red O staining. Many areas show no stained fat. Occasionally, however, an isolated hepatic cell is crowded with fat droplets. In one area there are many cells containing considerable fat in droplets are large as 16μ in diameter. These fatty areas make up less than one-half of 1% of the areas examined but within some of these areas the cells seem to have in them more than one-half as much fat as described in animal 72 which was starved for 96 hours. Most of the areas contain less fat than those illustrated in Grafflin's ('40) paper.

Cocoonut oil 15% emulsion, 1 cm^3 per hour for 24 hours; 5.45 gm per kilogram in 24 hours or 0.227 gm per kilogram per hour. Animal 60, figure 2.

In the hepatic cells of this animal there are many small droplets stained with Oil red O. Most of the droplets of fat are in the cytoplasm of the liver cells near the cell membrane adjacent to the sinusoids. The fat is quite evenly distributed throughout the lobules, but there is slightly less immediately about the central veins. Most of the droplets are less than 0.3μ in diameter. The larger droplets are 4.0 to 6.0μ in diameter. No evidence of fat emboli could be found. In the lumina of the blood vessels droplets of the emulsion similar to those injected can be seen. The droplets are for the most part less than 1.0μ in diameter. Few droplets more than 2.0μ in diameter are observed in the lumina of the vessels. The fat droplets within the hepatic cells are in the peripheral portion of the cytoplasm adjacent to the sinusoids, whereas there is much less fat in the cytoplasm immediately about the nuclei. The Kupffer cells at most contain only several small fat droplets.

Olive oil 10% emulsion, 1 cm³ per hour for 24 hours; 3.55 gm per kilogram in 24 hours or 0.148 gm per kilogram per hour followed by 24 hours rest. Animal 69, figure 3.

The droplets of fat in the hepatic cells of this animal vary considerably in size. The largest are about 6 to 8 μ in diameter, most of them are between 2 and 5 μ . The fat droplets are in the peripheral cytoplasm of the hepatic cells adjacent to the sinusoidal endothelium. No large droplets or evidence of emboli in the liver are found and no evidence of cellular infiltration or beginning of fibrosis is seen.

The amount of fat as indicated by the size and number of droplets stained is considerably more than that seen in well-fed, normal guinea pigs (Grafflin, '40). The nuclei are normal in appearance and there is not histologically demonstrable damage to the hepatic cells. The amount of fat seen in sections of the liver of this animal appears to be considerably more than that observed by Grafflin ('40), figures 2 to 5, page 485 of Grafflin's paper.

Cocoanut oil 15% emulsion, 2 cm³ per hour for 24 hours; 16.66 gm per kilogram for 24 hours or 0.694 gm per kilogram per hour. Animal 54.

There are a greater number of fat droplets most of which are larger than those seen in animal 60, 1 cm³/hour/24 hours. Most of the droplets are in the cytoplasm of the liver cells but a noticeable amount is also seen in the lumina of the sinusoids. The majority of the fat droplets are 2.0 to 6.0 μ in diameter. In the lumina of the blood vessels the droplets vary from less than 0.2 to as much as 4.0 to 6.0 μ in diameter but no larger masses of fat are seen. The nuclei are normal in appearance. The cytoplasm about the nuclei contains very little fat. The Kupffer cells contain very little fat.

Cocoanut oil 15% emulsion, 2 cm³ per hour for 24 hours; 16.37 gm per kilogram for 24 hours or 0.682 gm per kilogram per hour followed by 96 hours rest. Animal 55, figure 4.

The fat droplets in the hepatic cells of this animal are much fewer in number and somewhat larger than those in the livers of the animals described above. Most of them are

between 5.0 and 15.0 μ in diameter. The cytoplasm of the cells appears to have been displaced. The cell membranes are prominent. Each cell contains usually one to three or 4 large droplets. There are no marked changes seen in the nuclei of the hepatic cells or the blood vessels. The Kupffer cells have only a few droplets (0.2 to 2.0 μ). No Kupffer cells filled with fat are found. No evidence of proliferation of fibroblasts can be found.

Cocconut oil 15% emulsion, 2 cm³ per hour for 68 hours; 14.11 gm per kilogram in 24 hours, 39.98 gm per kilogram for 68 hours, or 0.588 gm per kilogram per hour. Animal 52.

In the hepatic cells there are many fat droplets from 2.0 to 6.0 μ in diameter. In the lumina of the sinusoids there are larger droplets, usually about 15 to 20 μ in diameter, and from 20 to 45 μ in length. There are several small areas 100 $\times$ 200 μ or larger where little fat is seen. In these areas the hepatic cell nuclei are pyknotic and there is considerable cellular infiltration. The Kupffer cells contain some fat but not a large amount. The fat droplets in the lumina of the blood vessel are larger than those seen in the animals described above. Some are as much as 8 to 12 μ in diameter.

Cocconut oil 15% emulsion, 2 cm³ per hour for 24 hours or 11.04 gm per kilogram, 0.460 gm per kilogram per hour followed by 24 hours rest; 250 mg choline each 12 hours. Animal 58, figure 5.

The fat in the hepatic cells is in droplets 2.0 to 8.0 μ in diameter. While there is somewhat less than was seen in tissues from animal 52 (68 hours, 2 cm³) there is a great deal. Most of the fat is in the peripheral portion of the cytoplasm. The nuclei of the hepatic cells are normal in appearance. In the lumina of the sinusoids there are many droplets of fat, as much as 20 μ in diameter and 25 to 40 μ in length. The Kupffer cells contain relatively little fat in droplets 0.2 to 2.0 μ in diameter. No evidence of cellular infiltration or fibroblastic proliferation can be found.

Starved 96 hours. No fat was injected into this animal. Animal 72, figure 6.

There are many fat droplets varying greatly in size some as much as 20μ in diameter. Most of the cytoplasm of the liver cells seems filled with the fat droplets. The fat is so abundant throughout the cytoplasm that the nuclei are difficult to observe. Only rarely can fat droplets be found in the lumina of the sinusoids. Practically all of the fat seen is within the hepatic cells. Very few droplets are observed in the Kupffer cells. In the lumina of some of the arteries a few fat droplets can be seen most of which are 0.5 to 2.0μ in diameter. There is somewhat less fat in the cells nearer the central vein than at the periphery of the lobules where other cellular constituents are obscured by the fat droplets present.

Pancreas

Pieces of the pancreas of each animal were fixed in formol-Zenker's, sectioned at 4μ , and stained with Azan. The islets of Langerhans were examined with special attention to the alpha cells. There were no noticeable changes in the alpha cells in any of the sections examined. It was considered that since the substance lipocin (Dragstedt, '36) has been described as an endocrine substance secreted by the pancreas, and since the alpha cells are the most numerous type in the islets other than the beta cells which secrete insulin, that it might be that the alpha cells could be the source of such a hormone. In the work of Homans ('15) and Allen ('22), changes were produced in the beta cells by the administration of glucose. It was on this basis that an attempt was made to produce changes in the alpha cells by the administration of a large amount of fat intravenously. It is possible that the duration of the injection in these experiments was not long enough. It was found that the amount of fat injected could not be increased without causing excessive accumulation of fat in the liver, lungs and spleen. If the experience with the earlier experiments with intravenous glucose are considered, the beta cells were affected by somewhat larger quantities of glucose over the same period of time, but it is not possible to draw any definite conclusion on that basis.

Spleen

Cocoanut oil 15% emulsion, 2 cm³ per hour for 24 hours; 16.66 gm per kilogram for 24 hours or 0.694 gm per kilogram per hour. Animal 54, figure 7.

The red pulp of the spleen of this animal is filled with numerous large fat droplets that obscure the other constituents of the red pulp. Many of these dropets are as large as $20 \times 40 \mu$. The relation between the fat droplets and the macrophages of the spleen is difficult to interpret. Many of the macrophages contain several small droplets of fat usually 0.2 to 3.0 μ in diameter. The larger fat droplets appear to be extracellular. The white pulp in which practically no fat is seen stands out in sharp contrast to the fat filled red pulp.

Cocoanut oil 15% emulsion, 2 cm³ per hour per 24 hours; 16.37 gm per kilogram for 24 hours or 0.682 gm per kilogram per hour followed by 96 hours rest. Animal 55, figure 8.

There is very little stained fat to be seen in this spleen. There are numerous vacuoles between 30 and 70 μ in diameter immediately around the Malpighian corpuscles; many of the Malpighian corpuscles appear to be completely surrounded by such vacuoles. There is a very small amount of fat adhering to the periphery of these vacuoles but the space within the vacuoles appears empty. This tissue was embedded in gelatin before sectioning and was mounted before staining to avoid the displacement of fat during manipulation of the sections. No anisotropic material could be found when sections of this spleen were examined with Nicol prisms. Within the red pulp there is practically no stained fat. There are occasional vacuoles as described above but very few fat droplets.

Arteries

Pieces of the aorta and heart were fixed in neutral 10% formalin, embedded in gelatin, sectioned at 10 μ , mounted and stained with Oil Red O and hematoxylin. In these sections droplets of fat could be seen in the lumen of the aorta, coronary arteries and in the vaso vasorum of the aorta. No fat droplets

or "lipophages" were found in the intima or media of any of these arteries. No droplets more than 8.0μ in diameter were seen in the vaso vasorum and no evidence of emboli could be found.

DISCUSSION

In these experiments from 148 to 694 mg of fat per kilogram of animal were injected intravenously. It appears from the examination of these tissues that the guinea pig does not metabolize fat rapidly enough to use, or store in the usual depots, as much as 150 mg of fat per kilogram of animal per hour without the deposition of neutral fat in the hepatic cells. In one experiment 21 mg of choline chloride per hour was administered. No decrease in the amount of fat in the hepatic cells was observed.

During the first 24 hours the fat appears in the hepatic cells as droplets 0.2 to 6.0μ in diameter. When the amount of fat is increased the number and size of the droplets increases. Later there is an accumulation of fat in the sinusoids of the liver. In animals given as much as 6.0 gm of fat per kilogram of animal per day there were no apparent degenerative changes in the hepatic cells in animals examined 96 hours after injection. When 16 gm per kilogram of animal per day were given there was vacuolation of the cytoplasm but no apparent degenerative changes in these cells. With 14 gm per 24 hours for more than 48 hours there was some cellular infiltration and evidence of proliferation of fibroblasts. The distribution of the injected fat was similar to that described by Brunshwig ('44) who injected fat emulsions into dogs.

In preliminary experiments with colloidal cholesterol as used by Bevans et al. ('48), the cholesterol appears to be readily phagocytized by the Kupffer cells while little of it is taken into the hepatic cells. The phagocytosis of cholesterol by the Kupffer cells and other reticulo-endothelial cells has been described by Leary ('41). The reticulo-endothelial system and lipid metabolism have been discussed by Jaffe ('38).

More recently lipid filled macrophages "lipophages" were discussed by Gordon ('47).

While there was an increase in the size of the droplets of fat to as much as $8.0\ \mu$ in diameter, during prolonged injection of neutral fat there did not appear to be any noticeable increase in the amount of lipid phagocytized by the reticulo-endothelial cells, that is, the Kupffer cells or the macrophages of the spleen. This may indicate that the nature of the lipid or more specifically the nature of the surface surrounding the lipid droplets as described by Knisely ('48) and Moreton ('48) and not the size of the droplets, Moreton ('47), is the more important factor in the phagocytosis of the more complex lipids in atheromatosis. No neutral fat was observed in the intima or media of the arteries examined.

CONCLUSION

When less than 6.0 gm of neutral fat per kilogram is injected into a guinea pig much of the fat accumulates in the hepatic cells and the spleen. Relatively little of this fat is taken up by the reticulo-endothelial cells. When larger amounts of fat are injected the hepatic cells are not able to take it up and fat accumulates in the sinusoids of the liver. The spleen becomes considerably enlarged by the accumulation of fat in it. No demonstrable changes occurred in the alpha cells of the islets of Langerhans. No fat or lipid was deposited in the intima or media of the arteries.

SUMMARY

Continuous intravenous injection of emulsions of neutral fat (tri-glycerides) 148 to 694 mg per kilogram per hour for 24 to 68 hours indicate that:

1. A large amount of this fat enters the hepatic cells.
2. Ninety-six hours after the injection of fat for 24 hours all but a small amount of this fat has left the liver without producing demonstrable changes in the hepatic cells.
3. Very little of the injected fat enters the Kupffer cells.

4. A large amount of fat injected accumulates in the spleen. Some, but not a large amount of this fat, is in the macrophages of the spleen.

5. Ninety-six hours after the injection of fat for 24 hours very little fat remains in the spleen.

6. When more than 6.0 gm per kilogram of animal is injected in 24 hours some degenerative changes occur in the hepatic cells, and fibroblastic proliferation can be observed.

7. No demonstrable changes occur in the alpha cells of the islets of Langerhans when as much as 16 gm of fat per kilogram of animal is injected each 24 hours for as long as three days.

8. No fat droplets or "lipophages" were found in the intima or media of the aorta or coronary artery.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

The optical system used for figures 1 to 8 consisted of a Bausch and Lomb fluorite (oil immersion) 43 mm, 40 $\times$ objective and a Zeiss Homal III (f equal 20 mm), with a bellows length of 80.2 cm which gave a magnification of 650 $\times$.

All of the tissues illustrated by these figures were fixed in neutral 10% formalin, kept in Baker's calcium-cadium formalin, cut at 10 μ stained with Oil Red O, and counterstained with Mayer's hematoxylin. The sections of the spleen were embedded in gelatin before sectioning.

1 Normal guinea pig liver — there is only one cell that contains any considerable amount of fat. Most of the areas of this section contained very little fat.

2 Guinea pig no. 60 — 5.45 gm of fat in 24 hours, numerous fat droplets 0.2 to 6.0 μ in diameter, practically all of which are in the hepatic cells.

3 Guinea pig no. 69 — 3.55 gm of fat intravenously in 24 hours, followed by 96 hours rest, the largest droplets are 6.0 to 8.0 μ in diameter. Most of the droplets are 2.0 to 5.0 μ in diameter.

4 Guinea pig no. 55 — 16.37 gm of fat in 24 hours followed by 96 hours rest. The cytoplasm of the hepatic cells has been displaced. There are fat droplets 5.0 to 15.0 μ in diameter in the hepatic cells.

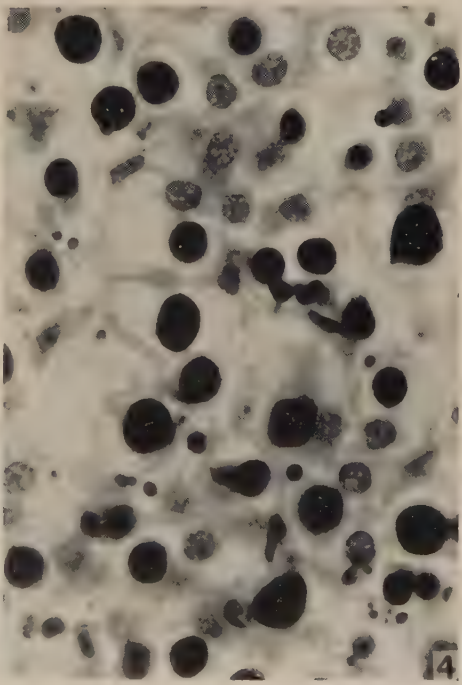
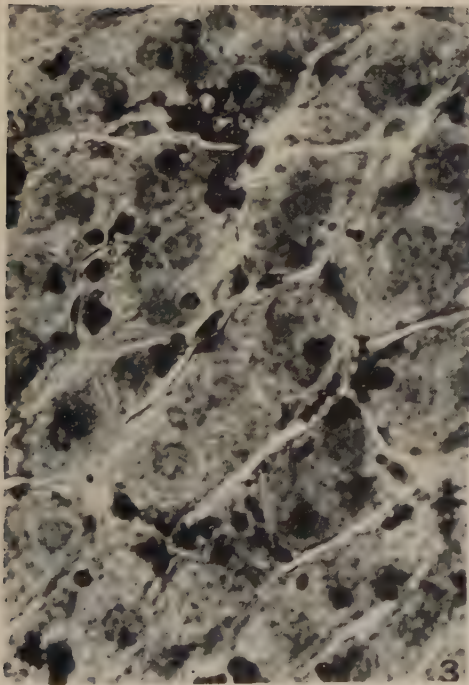
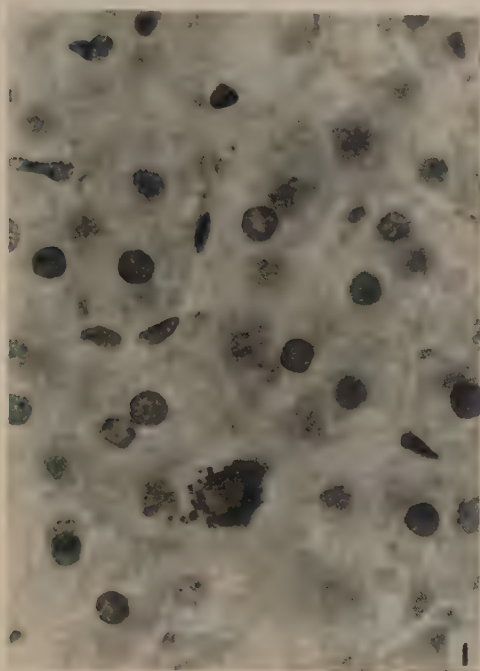


PLATE 2

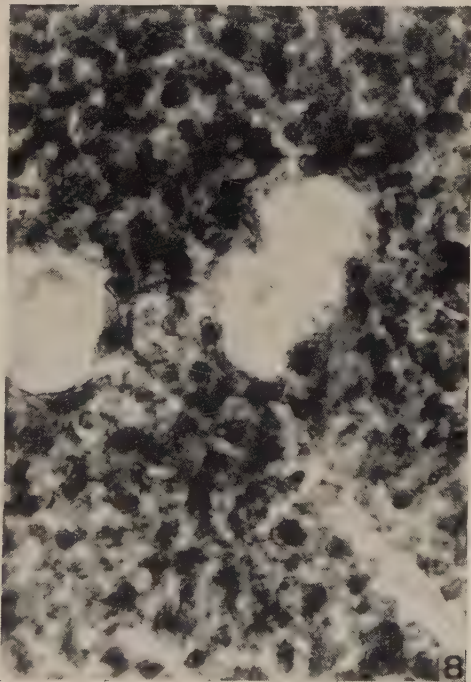
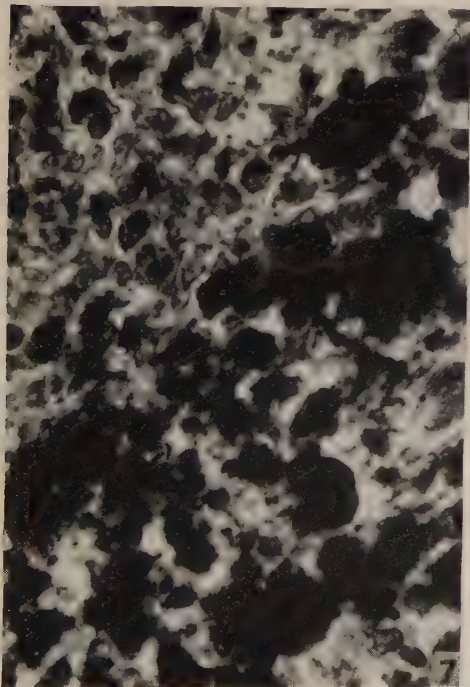
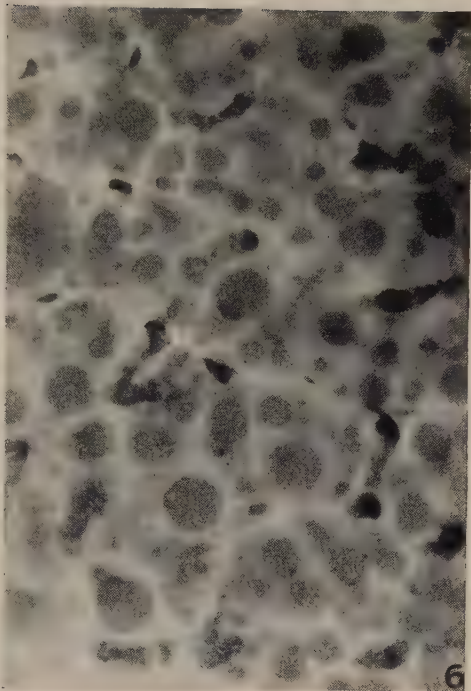
EXPLANATION OF FIGURES

5 Guinea pig no. 58 — 11.04 gm of fat intravenously in 24 hours with 250 mg choline chloride each 12 hours during and 24 hours after injection of fat. There are numerous droplets of fat 2.0 and 8.0 μ in diameter in the hepatic cells. There are numerous large fat droplets in the sinusoids.

6 Guinea pig no. 72 — no fat was injected into this animal. It was starved for 96 hours. There are numerous fat droplets, some of which are as much as 20 μ in diameter in the hepatic cells of the liver. Relatively few fat droplets can be found in the sinusoids.

7 Guinea pig no. 54 — 16.66 gm of fat intravenously in 24 hours. Spleen — in the upper left is white pulp, the lower right is red pulp. In the red pulp there are a great many large fat droplets many of which are from 20 to 40 μ in diameter.

8 Guinea pig no. 55 — 16.37 gm of fat in 24 hours followed by 96 hours rest. Spleen — the upper left is white pulp. The large vacuoles are just outside the Malpighian corpuscle. In the periphery of the vacuoles there is a very small amount of stained lipid. Practically no stained fat is seen in the red pulp.



HISTOCHEMICAL STUDY OF ALKALINE PHOSPHATASE IN TISSUE PREPARED BY THE FREEZING-DRYING METHOD

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SIX FIGURES

Gomori ('39) and Takamatsu ('39) have presented methods for histochemical demonstration of alkaline phosphatase. They found that alkaline phosphatase is not destroyed by fixation in alcohol (or acetone) or by imbedding in paraffin. Slides are prepared according to routine methods, deparaffinized, and incubated at 37°C. for one to two hours in a solution containing sodium glycerophosphate, CaCl_2 and sodium barbital at pH 9.3. During this period, the phosphatase releases phosphate which is precipitated as $\text{Ca}_3(\text{PO}_4)_2$. This substance is then converted to black cobalt sulphide. The areas where this black precipitate occurs are considered to be the sites where the enzyme alkaline phosphatase is located in the tissues.

The effect of fixation and embedding on the cytological localization of alkaline phosphatase has been studied in tissue sections by Danielli ('46) and Emmel ('46). Danielli ('46) concluded that no significant loss of alkaline phosphatase activity occurs as a result of fixation in ethyl alcohol and the cutting of frozen sections. The frozen sections which he used were cut from alcohol fixed blocks. The intracellular distribution of alkaline phosphatase has been studied in specimens prepared by the freezing-drying technique of Emmel ('46). However, he treated the dried tissue for 4 hours

with absolute alcohol to produce partial denaturation, then cleared in benzene and imbedded in paraffin. He concluded that tissues prepared by acetone-dehydration and the freezing-drying technique showed practically identical results in the intestinal epithelium and in the brush border of the renal proximal tubules.

Stafford ('48) pointed out that by Gomori's method 30–35% of the enzymatic activity is lost by the acetone fixation, while only 10–12% is lost if alcohol is used. It seemed to us very probable that if alcohol or acetone could be entirely avoided during the fixation, the true amount of alkaline phosphatase activity might be demonstrated more accurately.

In this work a comparative histochemical study of alkaline phosphatase in different tissues of the cat and dog was undertaken. Using both chemical fixation (80% alcohol) and the freezing-drying method as modified by our laboratory (Wang and Grossman, '49). In both cases the sodium glycerophosphate substrate of Gomori has been used for incubation ('41). This comparison revealed that alkaline phosphatase may be present in regions of tissues in which no alkaline phosphatase could be detected after chemical fixation.

METHOD

Tissue from dogs and cats was used. Pieces of liver, stomach, kidney, duodenum and pancreas were fixed in 80% alcohol. Other pieces from the same tissue were frozen in isopentane chilled in liquid air at $-190^{\circ}\text{C}.$, and dried in a vacuum system at $-31^{\circ}\text{C}.$ for about 5 days. The dehydrated tissues were infiltrated with paraffin by evacuation of the air. Histochemical study of alkaline phosphatase was carried out on the frozen-dried deparaffinized section and the alcohol fixed section of the same tissue. Both were mounted on one slide, side by side, and were incubated in Gomori's substrate (Gomori, '41) at the same time for one to two hours at $37^{\circ}\text{C}.$ and pH 9.3.

RESULTS

1. Kidney of dog. The distribution of renal alkaline phosphatase has been studied in different species of mammals (Bourne, '43, Gomori, '41), man (Kaba, '41), other vertebrates (Wilmer, '44), and in mouse and chick embryos (Kabat, '41, Moog, '44). In all cases alkaline phosphatase has been found to be highly concentrated in the proximal convoluted tubules

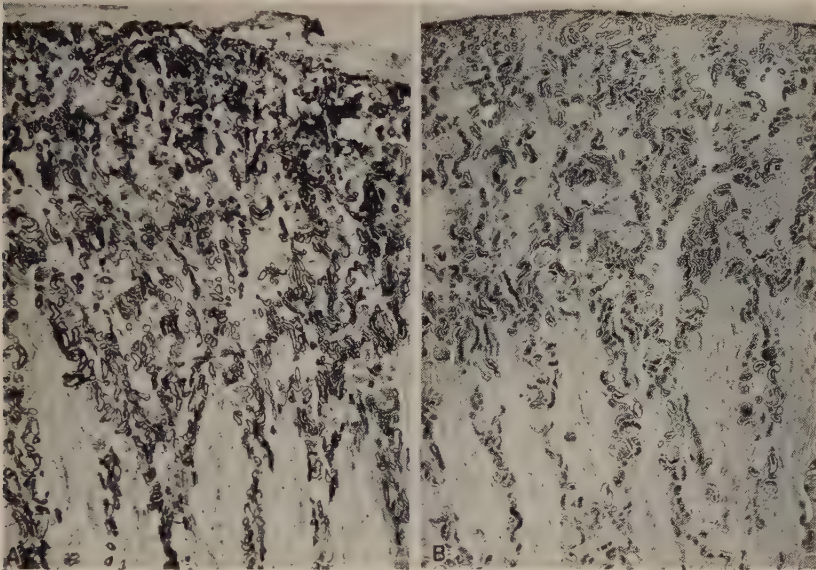


Fig. 1 Dog. Kidney. Comparison of alkaline phosphatase staining reaction with freezing-drying (A) and 80% alcohol fixation (B).

The alkaline phosphatase is demonstrable (1) in the glomeruli in (A) but not in (B), (2) in the brush border and lumen of the proximal convoluted tubules of both but more intensely in (A) than in (B), and (3) in the distal convoluted tubule in (A).

and especially in the brush border. The distal convoluted tubules are invariably negative. The ascending limb of Henle's loop in man and dog is also positive (Gomori, '41). The glomeruli are negative except in the cat (Gomori, '41). In the present experiments using the freezing-drying method, the reaction for alkaline phosphatase was found to be positive

in the glomeruli of the kidney of the dog and was more intense in the epithelium of the proximal convoluted tubules than with alcohol fixation. Besides, the alkaline phosphatase reaction was also positive in the distal convoluted tubules and Henle's loop by the freezing-drying method. In figures 1A and 1B the results obtained on the dog's kidney with the freezing-drying technique and with chemical fixation can be compared. In the latter alkaline phosphatase was found highly concentrated in the proximal convoluted tubules and especially

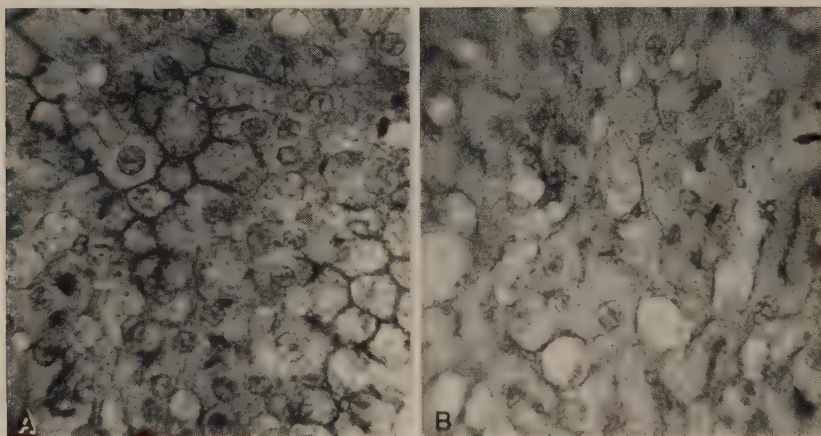


Fig. 2 Dog. Liver. (A) Frozen dried. (B) 80% alcohol fixation. (1) The staining of the cytoplasm of hepatic cells in (A) is more pronounced than in (B). (2) The nuclear membrane and karyoplasm in (A) shows more intense staining than in (B). (3) The bile capillaries are much more distinctly stained in (A) than in (B).

in the brush borders while in the former it was also found in the glomeruli. The epithelium of the proximal convoluted tubules stained more intensely than with alcohol fixation, and at times the distal convoluted tubules and Henle's loop were also positive.

2. Liver of dog and cat. Gomori was the first to notice marked phosphatase activity in the bile capillaries in the liver of some species. By Gomori's method ('41) the alkaline phosphatase activity is strongly positive in the bile capillaries

and the wall of the large bile ducts, but it is only very poorly shown in other structures. The cytoplasm of the liver cells take on a faint grayish color. Most of the nuclei are visible due to the staining of the nuclear membrane (fig. 2B). The endothelium of the sinusoids is only occasionally positive. The bile capillaries are outlined as fine black lines, sometimes showing a lumen (fig. 2B). Similarly treated sections prepared by the freezing-drying method showed several differences.

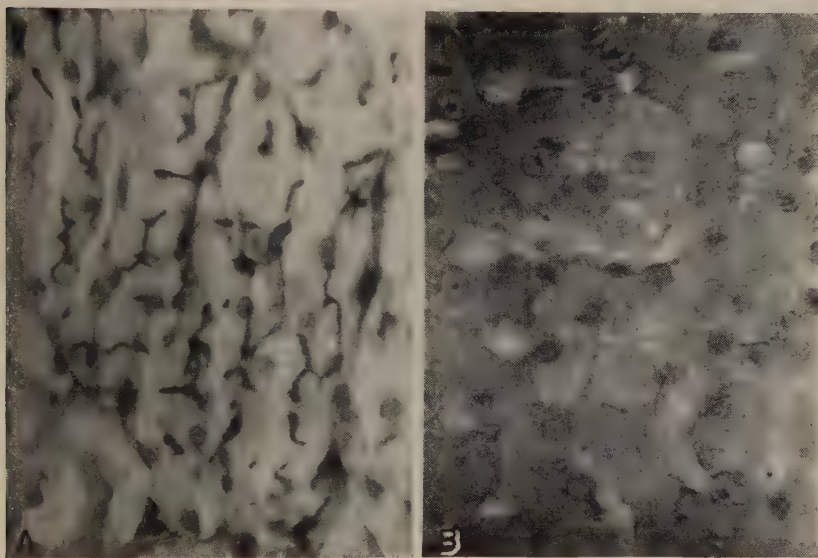


Fig. 3 Cat. Liver. (A) Frozen dried. (B) 80% alcohol fixation.

First, the cytoplasm shows more pronounced staining and the nuclei of the hepatic cells are definitely positive, both the nuclear membrane and the karyoplasm. Second, in contrast to the sections made by alcohol fixation, the bile capillaries are much more distinctly stained, so the demarcation between hepatic cells is accordingly very sharp. Third, a large amount of alkaline phosphatase activity is also seen in the hepatic ducts and the endothelial lining of the sinusoids. In figures 2A and 2B the results obtained with the freezing-drying technique and with alcohol fixation on the dog's liver

can be compared. In figures 3A and 3B a similar comparison for the cat's liver can be made.

3. Duodenum of the dog and cat. Histochemical studies have revealed that alkaline phosphatase is localized in the superficial intestinal epithelium, being more concentrated in the duodenum (Gomori, '41, Kabat, '41). Sections made by the freezing-drying method and by the alcohol fixative method

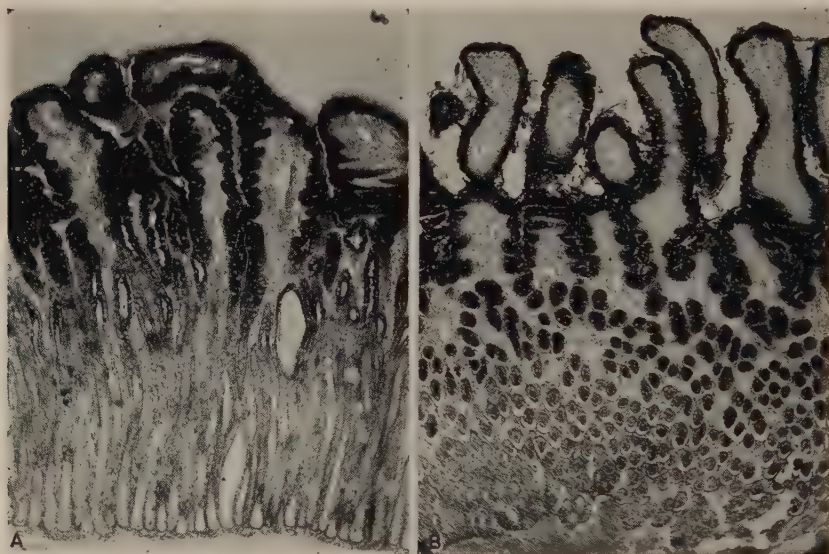


Fig. 4 Dog. Duodenum. (A) Frozen dried. (B) 80% alcohol fixation. The alkaline phosphatase reaction is prominent in the surface epithelial cells both in (A) and (B). The goblet cells are negative.

did not show important differences. Since the alkaline phosphatase is very concentrated in the duodenal epithelium (Gomori, '41), its lessened activity after chemical fixation cannot be detected (figs. 4A and 4B).

4. Stomach of the dog and cat. It has been shown (Gomori, '41) that the glandular epithelium of the stomach is negative in all species while the capillaries between the glands, especially those around the basal portion of the gland are strongly positive. In our experiments all these points have been con-

firmed by repeating Gomori's procedure, but by the freezing-drying method, the reaction becomes more strongly positive. In figures 5A and 5B the results obtained with these two techniques on the cat's stomach can be compared.

5. Pancreas of the dog. Similar results were obtained by the two methods, i.e., the acinar cells are negative, while the interlobular and intralobular ducts and centroacinar cells

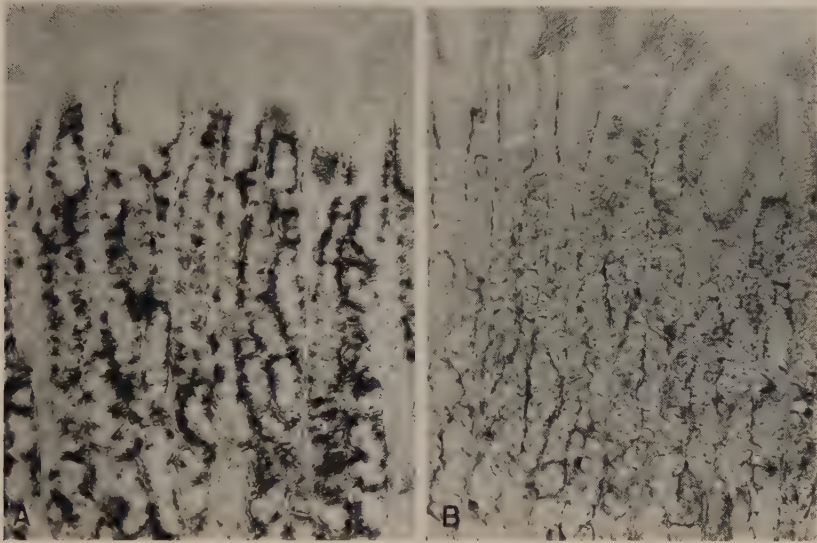


Fig. 5 Cat. Stomach. (A) Frozen dried. (B) 80% alcohol fixation. The stroma around the gland is positive both in (A) and in (B) but the reaction is more strongly positive in (A). The surface epithelium is negative in both cases.

stained positive, but by the freezing-drying method, the intralobular ducts and centroacinar cells were more intensely stained.

6. Pancreas of the cat. The pancreas of the cat has been reported to be entirely negative for alkaline phosphatase by Gomori ('41). By repeating his method we arrived at the same conclusion. However, by the freezing-drying method we found that the lining epithelial cells of the large ducts

and the blood vessels were slightly but definitely positive. The intralobular ducts and centroacinar cells were still negative (fig. 6).

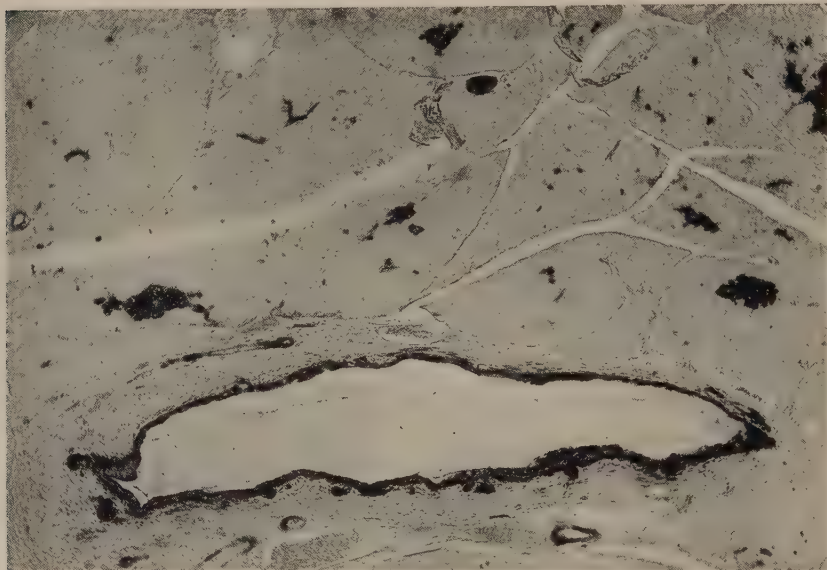


Fig. 6 Cat. Pancreas. (Frozen dried.) The epithelial cells of the large interlobular duct and the blood vessels are positive for alkaline phosphatase but the acinar cells, intralobular ducts and centroacinar cells are negative.

SUMMARY

1. A histochemical study of alkaline phosphatase was carried out in different tissues of the dog and cat comparing chemical fixation (alcohol) with fixation by freezing-drying.

2. With the freezing-drying technique, alkaline phosphatase appears in greater amounts than with chemical fixation.

CONCLUSION

From these investigations, it may be concluded that:

1. A part of alkaline phosphatase in the tissue is inactivated by chemical fixation.

2. If a tissue has a very high alkaline phosphatase content, the lessening of activity by chemical fixation cannot be detected by the freezing-drying method, and the results are the same with the two fixation methods, as in the case of the duodenum of the dog and cat. If the tissue has a moderate alkaline phosphatase content, its lessened activity by chemical fixation can be detected as indicated by the results obtained with the kidneys and liver of the dog and cat and the pancreas of the dog. If the tissue has a very slight alkaline phosphatase content, such as in the pancreas of the cat, it may give a completely negative result by alcohol fixation, but a positive reaction after fixation by freezing-drying.

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CONDITIONS EFFECTING THE PASSAGE OF SPERMATOOZOA THROUGH THE UTERO-TUBAL JUNCTION OF THE RAT¹

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In accounts of the transport of spermatozoa through the female reproductive tract of mammals, the conditions governing the passage of sperms from the uterus into the oviduct have not been adequately treated. Anatomical studies of the utero-tubal region of the genital tract indicate that, in many species, including the rat, a valve-like structure is present which interrupts a simple continuity of the lumen between the uterus and oviduct (Andersen, '28; Lee, '28; Kelly, '28; Alden, '43). This structure will be referred to as the utero-tubal "valve" in this paper. Physiological investigations to determine the factors controlling this structure have led to the employment of the technique of measuring the pressures required to force gas or fluids from the uterus into the oviduct (Lee, '28; Andersen, '28; Parker, '31; Alden, '43; Siegler, '44).

These methods are open to criticism because of their unphysiological nature and also because dead or anesthetized animals have been used. In some species, as the cow and sheep, relatively low pressures were sufficient to force fluid into the oviducts (Andersen, '28) while very high pressures were required to accomplish this in the rat (Alden, '43). In the rabbit, Parker ('31) claimed that passage of colored fluid from uterus to oviduct was obtained with ease, which is in contrast to the results reported by Andersen ('28).

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Our interest in this problem resulted from the observation that beef hyaluronidase when placed into the uterus of a rat preceding ovulation failed to enter the oviduct and subsequently denude the ova of their follicle cells (Leonard, Perlman and Kurzrok, '47). This finding led us to investigate some of the conditions which must prevail in order that spermatozoa may pass through the "valve" and enter the oviducts in the rat.

METHODS

The female rats used in these experiments were rigorously selected for estrus by the vaginal smear technique followed by testing for lordosis with male rats (Blandau, Boling and Young, '39). This was done between 7-9 P.M. and invariably those females which exhibited lordosis ovulated by 9 A.M. the following morning. Rat sperms were obtained from the epididymis and vas deferens and suspended in calcium-free Ringer's solution in some experiments, or were obtained from the uterine horns of bred rats in others. Mouse and guinea pig sperm suspensions were prepared in Ringer's solution as described above and bull sperms³ were used undiluted as obtained from ejaculates. The final volume of the prepared sperm suspensions did not exceed 1 cm³ per mouse, 2 cm³ per rat or 2.5 cm³ per guinea pig.

The sperms were injected through the wall of the uterus into the uterine horns in amounts of .15-.20 cm³ per horn. Whenever they were introduced while the rats were in heat, some of the uterine fluid was first withdrawn. In almost all cases ligatures were placed so that they could be drawn tight, as the needle was removed, to prevent leakage into the abdominal cavity or through the cervix. Ether was used as the anesthetic and it did not require more than 5 minutes to complete the operation.

Autopsies were made at several periods after insemination and extreme care was taken to prevent contamination of

³ Dr. Robert Bratton of the Department of Animal Husbandry kindly supplied us with the bull spermatozoa.

instruments and glassware with spermatozoa when the oviducts were examined for their presence. A small hemostat was clamped just at or slightly above the utero-tubal junction, holding the uterus with fine forceps in such a way as to force the uterine contents away from the tubes. The oviducts were cut free from the uterus close to the hemostat and each ovary with the attached oviduct was rinsed in a watch crystal of saline and then was transferred to a depression slide containing Ringer's. With the aid of a binocular dissecting-scope, each ovary was removed and the oviduct divided in half approximately mid-way along its length. Each half of the oviduct was placed on a microscope slide with a small quantity of Ringer's, cut in smaller pieces and each teased and pressed with needles to force out its contents. The slides were then examined under the compound microscope. Further details of methods will be described with the individual experiments.

EXPERIMENTAL

Passage of normal rat sperms

The first experiments were performed to determine if rat spermatozoa introduced artificially into the uterus of anesthetized rats could be recovered from the oviduct. Blandau and Money ('44) were able to recover them from 100% of the oviducts one hour after normal copulation. Five female rats in heat were inseminated with sperm suspensions obtained from the uterus of other bred females and all became pregnant. Four rats in heat were artificially inseminated with sperm suspensions prepared from the vas deferens and epididymis and one to two hours later few to many sperms were recovered in all oviducts. Some of them were still motile. It was noted also that those remaining in the uterus one to two hours following insemination were vigorously motile.

Three rats were bred and the oviducts examined 17-18 hours later. All of the ova recovered (21) were fertilized

and the only extraneous spermatozoa seen in the oviduct were those immediately in the vicinity of the mass of ova. The absence of large numbers of sperms in the oviducts 12-20 hours post-coitus in the rat has been noted many times in the course of other investigations in this laboratory. Spermatozoa were sometimes imbedded among the cumulus cells and were visible after dispersing the follicle cells with hyaluronidase *in vitro* (McClean and Rowlands, '42; Leonard and Kurzrok, '45). These observations led us to consider that one to 4 hours following insemination was the optimum time for examining the oviducts for the presence of sperm, although examinations were made in some instances after a longer interval.

Passage of rat sperms in post-estrous rats

In order to determine if rat sperms could pass the valve at times other than estrus, a series of rats were artificially inseminated 12, 24, 48, and 72 hours post-estrus. Some difficulty was encountered occasionally in forcing the suspension into the small muscular uterus and precaution was taken to prevent the back flow of liquid along the side of the needle. The results are summarized in table 1.

TABLE 1

Passage of rat sperms through the utero-tubal junction in post-estrous rats

EXP. NO.	TIME OF INSEMINATION POST-ESTRUS	LAPSE TIME BEFORE EXAMINATION	NO. OF OVIDUCTS EXAMINED	NO. OF OVIDUCTS WITH SPERM	CONDITION OF UTERINE SPERM AT AUTOPSY
	<i>hours</i>	<i>hours</i>			
1	12	11-12	8	1	dead
2	12	1-2	4	4	many motile
3	14	1-4	8	3	mostly dead or feebly motile
4	24	2.5-3	8	8	many motile
5	48	1.5-2	8	2	dead or very feebly motile
6	72	1.5-2	4	4	many motile

In the first experiment in which the sperms were injected 12 hours after heat and the oviducts examined after a lapse of 12 additional hours, none of the recovered ova were fertilized and most of the follicle cells remained about the ova. In only one oviduct were sperms seen, entrapped in the viscous material around the ova. Practically all sperms in the uteri were dead.

In the remaining experiments, at periods from 12-72 hours post-estrus, sperms were found in 21 of 32 oviducts but not frequently in large numbers when examined shortly after insemination. Although no quantitative measurements were made, it appeared that fewer spermatozoa went through the utero-tubal junction than when the rats were in heat. It was very noticeable, however, that the uterine sperms at the time of autopsy in this series lost their motility very rapidly, and it appeared that the uterine environment in diestrus was not conducive to longevity of the spermatozoa. There seemed to be no doubt, however, from these experiments that rat sperms passed from uterus through the valve into the oviduct in the diestrous period.

Passage of india ink

Although it had been noted that substances in solution did not pass unaided from the uterus to the tubes, it was considered possible that small, particulate matter might do so. To test this possibility 6 rats in heat were given intra-uterine injections of india ink diluted 1:3 with Ringer's. On examination under the binocular dissecting-scope 17 hours later no ink was visible in the oviducts except in one instance. The oviducts were sectioned and microscopic examination revealed no ink particles. That horn of the uterus whose oviduct contained ink was atypical in that no fluid was present in it at the time of injecting the ink. Examination of the oviducts of three additional rats three hours after administering ink also failed to show passage through the valves.

Passage of dead rat sperms

Since the failure of ink to pass the valve might have been due to the foreign nature of the substance, determinations were made on the ability of immotile rat sperms to pass into the oviducts under these conditions. Sperm suspensions were heated to 50°C. for 5–10 minutes until all motility ceased and were transferred to the uteri of rats in heat in the usual way. Ten rats in this experiment were examined 17–18 hours later and not a single spermatozoon was found in their oviducts. Six other rats similarly injected were examined one to two hours later and likewise none was found. Furthermore, when the sperms were removed from the uteri at autopsy and centrifuged, it was found that the hyaluronidase in the supernatant fluid was still active and that it was capable of denuding rat ova very quickly *in vitro*. In those experiments in which examination was made 17–18 hours after insemination the ova recovered from these rats were not denuded, indicating that little if any hyaluronidase passed through, although it was still present in the uterus. It appeared that substances could not go through the valve passively.

Passage of foreign sperms

So far, the experiments indicated that only motile rat sperms were able to negotiate the passage into the oviducts and it was considered possible that motility *per se* was sufficient to permit passage through the valves. On this assumption, foreign motile spermatozoa should pass through. Only two previous investigations on the passage of foreign sperms from uterus to oviducts could be found. Philips and Andrews ('37) reported that in three of 7 ewes, rat sperms traveled from the uterus into the oviduct at a rate only slightly less than ram sperms. Yochem ('29) studying guinea pig sperm survival in female rats indicated in his data that in 11 of 18 oviducts, foreign sperms were found which appeared to have migrated from the uterus to the oviduct.

In view of these results experiments similar to Yochem's were carried out using bull, mouse, and guinea pig sperm.

The results of these experiments are given in table 2. Mouse spermatozoa were unable to pass into the oviducts except in one case. Those remaining in the uterus at autopsy had fair to poor motility. Bull sperms also failed to pass through the utero-tubal junction although in three oviducts of 14 examined a few were found. In two of these three cases only one dead sperm was seen, the other had several

TABLE 2
*Failure of foreign sperms to pass through utero-tubal
junction of the rat in estrus*

EXP. NO.	SPECIES OF SPERM	LAPSE TIME BEFORE EXAMINATION	NO. OF OVIDUCTS EXAMINED	NO. OF OVIDUCTS WITH SPERM	REMARKS
		<i>hours</i>			
1	Mouse	12-13	4	0	
2	Mouse	1.5-2	4	1	1 sperm
3	Mouse	1.5-2	4	0	
4	Bull	1.5-12	6	2	In same rat; 1 sperm and several sperm/oviduct
5	Bull	1.5-3	8	1	numerous dead sperm
6	Guinea pig	2-12	10	0	
7	Guinea pig	12-14	14	0	

motile ones present. The bull sperms remained motile in the uteri for three hours and longer after insemination.

The results obtained with the guinea pig sperm were the same as above, only in every instance not one was found in the oviducts. In the experiments where examination was made 12-14 hours after insemination the exact technique as described by Yochem ('29) was employed in insemination and the time of examination of the oviducts. Guinea pig spermatozoa maintained their motility in the rat's uterus

better and longer than either mouse or rat sperm. Therefore it appeared that motility *per se* was not the only factor necessary for passage of sperms through the utero-tubal valve.

Passage of combined foreign and rat sperms

In the light of the preceding results it seemed possible that the utero-tubal valve might be relaxed by some chemical stimulation from the rat sperm. If this were so then motile, foreign sperms in the vicinity of the junction might conceivably pass into the oviducts along with the motile rat sperms. To test this hypothesis, motile guinea pig, mouse, and bull spermatozoa were combined with rat spermatozoa in the uteri of a number of rats and the oviducts examined.

It was discovered that rat sperms were quickly immobilized in mixtures with bull semen. By suspending the bull semen in 5 volumes of Ringer's, centrifuging the sperm carefully and resuspending them in Ringer's, it was found that the deleterious effect on rat sperm motility was eliminated.

In the first two experiments in table 3 the rat and guinea pig sperms were combined *in vitro* and placed into the uterus. In the remaining ones the foreign sperms (heavy suspensions) were first introduced into each uterus with a fine hypodermic needle but no ligatures were made. The females were then placed with the males as soon as they recovered from the anesthesia, and they all copulated within a half-hour. Examination was made for motility of the uterine sperms at the time the oviducts were removed.

The results in table 3 show that, with the exception of one experiment, very few foreign sperms passed through the utero-tubal valve. Rat sperms, in all experiments, were recovered in the oviducts. Many experiments were done using guinea pig sperm because of their long maintenance of motility and their availability. At autopsy, motility of all sperm types in the uteri was good. In the one experiment,

where a high percentage of oviducts contained guinea pig sperms, the clamp was placed far down towards the uterus and it was felt that contamination from the pocket of sperms in the apices of the uteri accounted for this result.

TABLE 3

*Recovery of foreign sperms from oviducts when combined with rat sperms in the rat uterus*¹

EXP. NO.	NO. OF RATS	NO. OF OVIDUCTS WITH RAT SPERM	NO. OF OVIDUCTS WITH FOREIGN SPERM	REMARKS
<i>Guinea pig sperm</i> ¹				
1	6	12	2	1 sperm; 1 clump of 3
2	7	14	10	sperm/oviduct, 1, 3, 4, 4, 2, 3, 1, 2, 1, 9
3	6	12	0	
4	6	12	1	1 sperm
5	6	12	2	1 sperm/oviduct
<i>Mouse sperm</i>				
6	4	7	0	
<i>Bull sperm</i>				
7	6	12	0	

¹ Oviducts examined 2-3 hours after insemination; motility in all sperm in uteri at autopsy.

DISCUSSION

In these experiments a new approach was made to the problem of the conditions which must prevail for spermatozoa to go from the uterus to the oviduct through the utero-tubal valve. The older methods which in general determined the pressures required to force the valve seemed unreliable. In some preliminary studies on rabbits this method was tried. Uterine fistulas were prepared (Reynolds, '30) on 4 rabbits and the amount of air pressure to force the valves varied from 10-200 mm of mercury in the unanesthetized animals when in heat. This variability plus the fact that they easily succumbed to what appeared to be air embolism led us to abandon this procedure.

In confirming the work of Blandau and Money ('44), that rat sperm pass into the oviduct within one hour after copulation or artificial uterine insemination, we failed to confirm the work of Farris ('42) who states that rat sperm do not pass into the tubes until ovulation time. In fact it was observed that they can pass through the valve at several periods during the estrous cycle. This is evidence that any hormonal control of the opening or closing of the valve correlated with a propitious time in the cycle is not absolute. No quantitative studies were made as to the relative number which passed through during these stages of the cycle. It did appear that motility and survival of rat sperm in the uteri of diestrous rats were not too satisfactory and that survival time of rat sperm suspensions prepared in Ringer's was quite variable. In some cases, however, as many sperms were found in the oviducts of diestrous rats as in those of artificially inseminated rats in heat.

The failure of india ink particles to pass through the valve eliminated the possibility that small particles might, by local stimulation, induce the valve to open. Also, it would seem possible that if the peristaltic waves passing over the uterus and oviduct were synchronized at some moment, then the valve would be relaxed to the extent of letting some ink through. This did not occur, or, if it did it could not be detected by the method employed.

The possibility of passive diffusion of particles through the valve seemed more remote when dead rat sperms, which had been placed in the uterus, were not found in the oviduct. The experiment was tried because it most closely approached natural conditions. Also, the possibility remained that some chemical substance associated with the sperms might cause a relaxation of the valve to permit some cells to be passively carried into the oviducts. Immobilization of the sperms was performed gently and without inactivating at least one enzyme associated with them (hyaluronidase). From these results it was concluded that *motility* was necessary for sperms to get past the barrier of the valve.

This conclusion suggested that if motility was an important factor then any small, motile cell might also pass through the junction. Foreign sperms were used to test this hypothesis. Yochem ('29) had already reported the necessary experiment of placing foreign sperms (from guinea pigs) into the rat uterus and recovering them from the oviduct. However, his results could not be confirmed nor were bull or mouse sperms able to pass the utero-tubal barrier even though, in all cases, they were still motile in the uteri at the time of autopsy. Thus it was realized that motility alone was not enough to insure the movement of sperms into the oviduct.

It was thought that if both rat and foreign sperms were combined in the uterus then whatever action living rat sperms exerted on the relaxation of the valve might also permit foreign sperms to go through at the same time. In spite of one experiment which was contrary to the other results, only rat sperms moved past the valve. This is a remarkable fact when it is realized that the rat sperms had to move through the many foreign sperms already in the uterus before passing through the valve, and yet leave the motile, foreign sperms behind.

Alden ('43), in a brief abstract, described the anatomical relationship of the oviduct to the uterus in the rat and reported no cyclic change in the characteristics of the mucosal fold which forms one wall of the tube as it enters the uterus. We have examined sections of this region and also noted that a free end of the oviduct projects into the lumen of the uterus for short distance. Alden suggests that proper tactile stimulation of the oviduct causes relaxation of the junction and that chemical irritants produce the opposite result. In the experiments reported here, tactile stimuli were present in the motile, foreign sperms, and if any chemical substances were necessary to relax the valve they should have been provided by the living rat sperms. It is difficult to visualize a temporary relaxation of the valve to allow passage of sperms in view of these experiments, but if it did do so, some

special feature of the rat sperms quite difficult to conceive must also be present. It is remarkable that so few rat sperms pass through into the oviducts normally while in the uterus they number in the millions. This observation has been made numerous times in the past and recently expressed on a rough quantitative basis by Blandau and Odor ('48). It is likely that the valve is responsible for this distribution of the spermatozoa.

It might be thought that the size and shape of the sperm would bear some relation to their ability to pass through the valve. Guinea pig sperms are slightly wider at their greatest diameter than rat sperms, but those of the bull and mouse are definitely smaller. The shape of the rat sperm is uniquely different with its thin, hooked head, and long tail. These features together with the possibility of a more powerful whipping action of the flagellum might be the means whereby only rat sperm force their way through the folds of the mucosa which close the opening of the oviduct. The valve might well be an adaptation not only to keep out foreign material but also to exert a selective action on the rat sperm. A premium would be placed on strong and prolonged motility.

Quite likely there is a species difference with regard to the functioning of the utero-tubal valve. In the rabbit Parker ('31) believes the sperms migrate up the uterus primarily by their own effort but probably are materially aided by the currents and counter-currents from the cilia in the tubes and the adjacent end of the uterus, which sweep them through the valve into the oviduct. Rat sperms, on the other hand, reach the valve passively in less than a minute but must go through the valve by their own locomotion. It may be that the uterus of the rabbit selects the most vigorous sperms, the final stage of the journey being aided by ciliary currents. The valve in the rat acts similarly to the rabbit's uterus in selecting vigorous sperms and in addition has a selective action on what else goes through. Yochem ('29) reported rat sperms migrating through the utero-tubal junction in the guinea pig and this was verified in one guinea pig tried.

Also Philips and Andrews ('37) found rat sperms migrating from the uterus to oviducts in the ewe in the presence of ram sperms. A comparative study of the behavior of the utero-tubal valve in different species by this technique would be very enlightening.

SUMMARY

A study was made on the function of the utero-tubal "valve" in the rat by placing inert particles, rat sperms, and foreign sperms into the uterus and noting their recovery in the oviducts after an optimum period of time. It was found that the valve permits the passage of living rat sperms during diestrus at periods from 12-72 hours after heat. The utero-tubal valve will not permit inert particles (india ink or immobilized rat sperms) to be passively carried into the oviduct. Motility is a necessary quality for rat sperms to pass through the valve. Motility alone, however, is not the only quality which will permit passage of sperms through the valve as bull, mouse, and guinea pig sperms cannot do so. Evidence is presented that there is little likelihood of a local chemically-stimulated relaxation of the valve to allow sperms through because mixtures of living rat and foreign sperms resulted in only rat sperms passing through the valve. The rat utero-tubal valve is apparently highly selective for the vigorous rat sperms and the species selectivity may be correlated with the morphology and activity of the sperm itself.

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NUCLEIC ACIDS AND THE FEULGEN REACTION

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TWELVE FIGURES

INTRODUCTION

Nucleic acids are usually recognized from the properties of their purine and pyrimidine components, or from the properties of their sugar components. The pyrimidine and purine components may be recognized in tissues by their characteristic absorption of ultraviolet light. This method of recognition does not distinguish between ribonucleic acid and desoxyribonucleic acid; distinction may be made on the basis of the difference in properties of ribose and desoxyribose as in the Feulgen nuclear reaction.

The Feulgen reaction has been used extensively as a histochemical test for desoxyribonucleic acid. Milovidov ('38) listed 458 references to investigations in which the technic had been used. Most of the investigators who are familiar with the technic accept it as specific for desoxyribonucleic acid; however, some, notably Stedman and Stedman ('43, '47), Choudhuri ('43) and Carr ('45), question such specificity.

Those who are familiar with the Feulgen technic recognize that the staining results with sections may vary greatly. The technic involves a series of chemical reactions some of which are not so clearly understood, a change in any one of which might have considerable influence on the final results.

Some studies of the Feulgen technic, particularly in regard to the effect of HCl hydrolysis on cells and sections of tissue, and the influence of alterations in the technical procedure on the final results, have been made in this Laboratory. The

results are presented here in the belief that they may be of interest to others.

MATERIALS AND METHOD

The Feulgen stain used was prepared according to the method described by Stowell ('46). The basic Fuchsin was obtained from Allied Chemical and Dye Corporation and bore certificate No. N.F. 54 of the Commission of Standardization of Biological Stains.

Desoxyribonucleic acid was prepared from calf thymus by the method of Mirsky and Pollister ('46) and was nearly pure as the ratio of N:P approached closely to the theoretical value. The concentration of desoxyribonucleic acid in the solutions varied considerably depending on the experiment; in some experiments the concentration was approximately 0.04%, in others greater.

Tissues used for sections were fixed in Carnoy's fixative (3 parts absolute ethyl alcohol and 1 part glacial acetic acid).

Ultraviolet light photomicrographs were made by the method previously described (Ely and Ross, '48a). Densities of film images were measured by means of a microdensitometer. (A modification of the microdensitometer described by Enns, '46, and Ely and Ross, '48b.)

Effects of HCl hydrolysis on sections of tissue, cell suspensions and on solutions of desoxyribonucleic acid

The characteristic high absorption of ultraviolet light of wave length 2600 Å by the nucleic acids was utilized to study the effects of HCl hydrolysis on the nucleic acid content of tissue sections and of cells in suspension. Sections 3 μ in thickness were mounted on quartz slides without adhesive, hydrolyzed for various lengths of time, and then photomicrographed with ultraviolet light. The negatives, or prints of the negatives, were examined to determine the amount of ultraviolet light absorbing material removed by hydrolysis. The sections were later stained by the Feulgen technic and

examined microscopically to compare the effect of hydrolysis on the intensity of the Feulgen stain.

Suspensions of unfixed and of Carnoy fixed cells (Walker Carcinoma No. 256 of the rat and also rat thymus) were prepared from fresh tissue in 0.85% NaCl solution, or from fixed tissue, by mincing with scissors and grinding with a glass rod in a porcelain crucible. Coarse particles were removed by filtration of the suspensions through cotton. The cells were hydrolyzed, and were stained in test tubes; reagent and dehydration solutions were removed by centrifugation and decantation. After completion of hydrolysis, or completion of the staining procedure, the cells were mounted on slides for microscopic examination or for ultraviolet light photomicrography. Extinction coefficients, when desired for accurate comparison, were calculated from data obtained by densitometric measurements of images on film; otherwise comparisons were made by microscopic examination.

If mild hydrolysis of desoxyribonucleic acid removes purines and not pyrimidines from the nucleic acid molecule and the degree of polymerization is not reduced too much it should be possible to separate the purines by dialysis. The constituents of the residue should have a lower N:P ratio since the purines are comparatively high in nitrogen content and contain no phosphorus. This was tested by determination of the N:P ratio in the residue and dialyzate of nucleic acid solutions that had been hydrolyzed and then dialyzed against distilled water through cellophane ("Nojax" casing No. 20/32, Visking Corporation, Chicago).

Determination of the approximate size and composition of the fragments produced by hydrolysis of desoxyribonucleic acid was accomplished by dialysis, ultracentrifugal sedimentation data, electrophoresis and estimations of the inorganic and organic content of hydrolyzed solutions.

Hydrolysis as carried out in the Feulgen technic removed from isolated cells and from sections of tissue the ultraviolet light absorbing material. These absorbing constituents are recognized to be the purine and pyrimidine bases of nu-

cleic acid (the comparatively little absorption by protein may be disregarded). It may be seen in figures 3-8 that removal of these constituents progressed as the length of time of hydrolysis was extended. Figure 3 illustrates the intense cytoplasmic and nuclear absorption in the crypts of Lieberkühn in unhydrolyzed sections of the duodenum of the rat. It may be seen in figure 4 that one minute hydrolysis removed most of the cytoplasmic ribonucleic acid (or the absorbing constituents of it) and left the nuclei clearly visible. Three to 6 minutes' hydrolysis apparently removed all of the ribonucleic acid of the cytoplasm, (figs. 5 and 6). After 20 minutes hydrolysis there was very little nuclear absorption; apparently the purine and pyrimidine constituents of desoxyribonucleic acid had been removed (fig. 8). The length of time necessary to remove all nuclear absorbing constituents was usually greater (up to 50 minutes) than in this experiment. Cytoplasmic absorbing constituents were always quickly removed.

Isolated cells of rat thymus, either unfixed or fixed in Carnoy's fixative, when hydrolyzed in 1 N HCl at 60°C. showed progressive removal of ultraviolet light absorbing constituent as the length of time of hydrolysis was extended. A graphic representation of the changes in extinction coefficients is shown in figure 1.

The constituents removed from sections by hydrolysis absorbed light intensely at 2654 Å. The residue remaining after evaporation of the hydrolyzate stained partly green and partly pink with the pyronin-methyl green stain.

When cell suspensions were hydrolyzed it was found that the typical Feulgen color developed in the hydrolyzate as well as in the cell nuclei. The color intensity in nuclei of thymus cells increased with time of hydrolysis to a maximum between 20 and 30 minutes' hydrolysis. The results of a typical experiment are shown in figure 2 A. Likewise the typical Feulgen color which developed when the reagent was added to HCl hydrolyzates of fixed thymus cells increased in intensity to a maximum after 24 to 30 minutes' hydrolysis, then declined rapidly (fig. 2 B). Similar curves of color intensity

were obtained with hydrolyzed desoxyribonucleic acid solutions (fig. 2 C).

When solutions of desoxyribonucleic acid were hydrolyzed at 60°C. in 1 N HCl for periods from one to 60 minutes and then placed in cellophane tubes and immersed in Feulgen's reagent, color developed inside the tube but not in the dialyzate during three hours at room temperature. The dialysis assemblies were then kept at 7°C. for an additional 18 hours. Only a

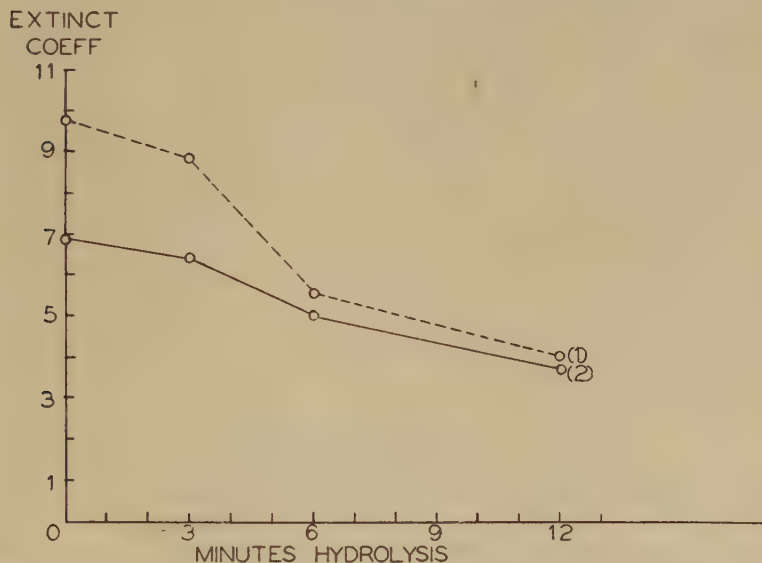


Fig. 1 Effect of Feulgen hydrolysis (1 N HCl, 60°C.) on the amount of ultra-violet light absorbing material in nuclei of isolated thymus cells. (1) Fixed cells; (2) Unfixed cells.

slight color developed in the dialyzates of solutions hydrolyzed for 30, 40 and 60 minutes; no color developed in the hydrolyzates of solutions hydrolyzed one, three, 6, 10 and 15 minutes. Unfixed rat thymus cells in suspension were similarly treated after 20 minutes' hydrolysis. The contents within the cellophane tube became colored, but the dialyzate did not.

Ultracentrifugal sedimentation of a hydrolyzed solution of desoxyribonucleic acid (30 minutes' hydrolysis, 1 N HCl, 60°C.) yielded results indicating that particles of various

sizes corresponding to molecular weights between 15,000 and 30,000 were present. Electrophoresis in the Tiselius electrophoresis apparatus failed to effect a separation of the components but indicated a mixture of particles differing somewhat in charge.

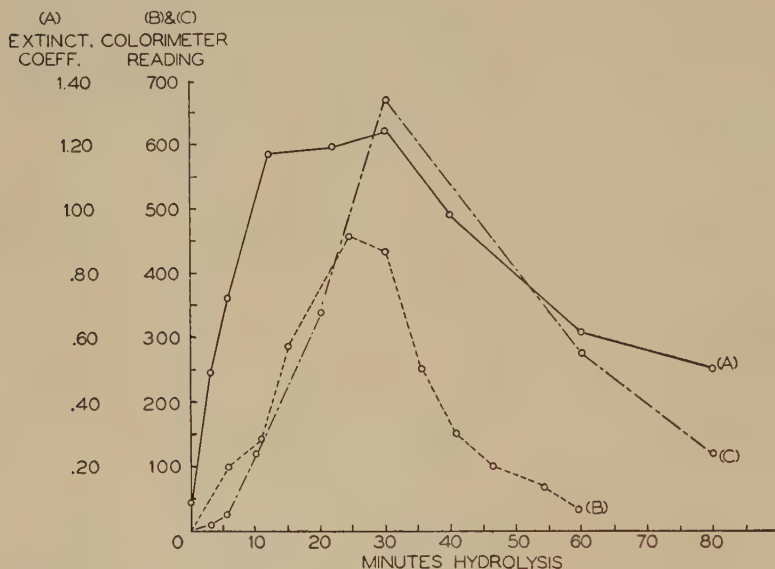


Fig. 2 Changes in Feulgen color intensity after hydrolysis of various lengths of time.

A. Extinction coefficient of Feulgen stained thymus nuclei.

B. Colormetric readings of Feulgen color intensity in hydrolyzate of thymus cells used in A.

C. Colorimetric readings of Feulgen color in hydrolyzed solution of desoxyribonucleic acid.

Ultraviolet light absorbing material was present in the dialyzate of hydrolyzed desoxyribonucleic acid but no phosphorus was found. Some nitrogen containing constituents small enough to pass through cellophane membranes were removed by hydrolysis. These were probably purines since they absorb ultraviolet light.

Nitrogen and phosphorus determinations on hydrolyzed desoxyribonucleic acid and on the dialyzate and residue of

such hydrolyzates, yielded the results shown in table 1, which may be interpreted in the following manner. Dialysis removed N but not P from nucleic acid solutions which had been hydrolyzed for 30 minutes, and lowered the ratio of N:P; the loss of N and the reduction of N:P were greater the longer the dialysis. Hydrolysis for this period of time, therefore,

TABLE 1
*Nitrogen:phosphorus ratios of hydrolyzed desoxyribonucleic acid,
dialyzate and residues*

HYDROLYSIS IN 1 N HCl at 60°C.	PHASE	DEGREE OF DIALYSIS	N:P RATIO	REMARKS
30 min.	Hydrolyzate	Partial	1.026	Dialyzed against a small vol. distilled H ₂ O
30 min.	Hydrolyzate	Complete	0.715	Dialyzed against a small vol. distilled H ₂ O
30 min.	Dialyzate	Incomplete	N present but no P	Dialyzed against a small vol. distilled H ₂ O
5 hrs.	Hydrolyzate	Complete	1.077	
5 hrs.	Dialyzate	Incomplete	2.04	
0	Dialyzate		No N and P present	
0	Residue		1.523	

liberated no inorganic phosphorus and produced no phosphorus-containing fragments small enough to pass through cellophane. After hydrolysis for 5 hours, the N:P ratio was reduced only by prolonged dialysis; the dialyzate contained organic P, but no inorganic P, in addition to N, and a high ratio of N:P. Hydrolysis for 5 hours, therefore, removed from the molecule both N-containing and P-containing fragments small enough to pass through cellophane. Untreated nucleic acid lost neither N nor P during dialysis.

The above results indicate that hydrolysis of sections or cells removes the constituents of nucleic acids, the amount removed depending on the length of hydrolysis. If this were true the amount of mineral constituents remaining in sections after hydrolysis should be reduced since the phosphorus of nucleic acids constitutes so large a part of the mineral constituents of tissues. Microincineration studies of hydrolyzed sections showed that one minute hydrolysis reduced the amount of ash in the cytoplasm of cells in the crypts of Lieberkühn of the duodenum of the rat and that as hydrolysis was continued 5, 10, 30 minutes (figs. 9-12) there was a gradual reduction of the quantity of ash until after 30 minutes there remained very little ash even in nuclei.

Hydrolysis was found to affect also staining with basic dyes such as toluidine blue, basic fuchsin and hematoxylin. These dyes stain not only the nuclei of cells but also the basic constituents of cytoplasm. After hydrolysis cytoplasmic staining decreased rapidly with time of hydrolysis. Nuclear staining also decreased but more slowly than did the cytoplasmic staining.

Influence of alterations in the technic on color intensity in the Feulgen reaction

Influence of concentration of HCl used for hydrolysis. When desoxyribonucleic acid was hydrolyzed for 30 minutes at 60°C. in HCl varying in concentration from 1 N to 1.33 N the color developed after addition of Feulgen's reagent decreased rapidly with increased concentration of acid. (See table 2.) However, when the excess of HCl above 1 N was neutralized before addition of reagent the decrease in color intensity did not occur (table 3). Apparently the decreased color intensity was caused largely by the presence of increased concentration of HCl above 1 N during color development rather than during hydrolysis.

The intensity of color developed in sections stained by the Feulgen procedure varied greatly with the concentration of HCl used for hydrolysis. Maximum stain intensity occurred

in sections of rat duodenum after 20 minutes' hydrolysis with 1 N HCl; after three, 6 and 12 minutes' hydrolysis with 1.5 N and after 20 to 30 minutes when 0.5 N acid was used. Those hydrolyzed with 0.5 N stained well after 60 minutes' hydrolysis.

When sections were hydrolyzed in various concentrations of HCl (0.5, 0.8, 1.0, 1.2, 1.5) for 10 minutes the greatest intensity of color appeared in the sections hydrolyzed in 1 N

TABLE 2

Influence of concentration of HCl on color intensity in the Feulgen reaction

ADDITIONAL 2 N HCl ADDED	TUBES WITH ACID ADDED		TUBES WITH DISTILLED H ₂ O ADDED	
	Normality of HCl	Colorimeter reading	Normality of HCl	Colorimeter reading
<i>cm</i> ³				
0.0	1.00	159	1.00	170
0.2	1.10	102	0.91	170
0.4	1.17	71	0.83	170
0.6	1.23	49	0.77	145
0.8	1.29	36	0.71	135
1.0	1.33	21	0.69	154

TABLE 3

Influence of concentration of HCl on color intensity in the Feulgen reaction

NORMALITY OF HCl FOR HYDROLYSIS	COLORIMETER READINGS	
	Excess HCl above 1 N neutralized after hydrolysis	Excess HCl above 1 N not neutralized
1.00	133	129
1.10	151	155
1.17	140	146
1.23	145	135
1.29	130	127
1.33	122	118

acid. When hydrolyzed for 12 minutes maximum color appeared in those hydrolyzed in 0.8, 1.0 or 1.2 N HCl (no appreciable difference); when hydrolyzed for 20, 24 and 30 minutes maximum color occurred in those hydrolyzed in 0.5 N acid but after 30 minutes' hydrolysis in 0.5 N HCl the maximum color was faint and diffuse and much less intense than after 10

minutes' hydrolysis in 1.0 N HCl. Hydrolysis for 10 minutes in 0.8 N HCl yielded approximately the same color intensity as 20 minutes' hydrolysis in 0.5 N.

When aliquots of a solution of desoxyribonucleic acid were hydrolyzed for various periods of time up to 1 hour maximum intensity of color developed after 40 minutes hydrolysis in 0.5 N HCl and after 10 minutes' hydrolysis in 1 N HCl.

Influence of time allowed for color development on intensity of color. When nucleic acid solution was hydrolyzed for 30 minutes at 60°C. in 1 N HCl (1 cm³ N.A. solution, 1 cm³ 2 N HCl; after hydrolysis 2 cm³ Feulgen's reagent) color developed to a maximum in 70 minutes then gradually declined to a low value in 5 hours.

Sections of tissue hydrolyzed for 24 minutes at 60°C. showed no appreciable difference in intensity of color when stained for 15 minutes to 6 hours. Sections stained for 24 hours were less intensely colored and staining was not sharply limited to nuclei; it appeared as if some diffusion of the stained material had occurred.

Influence of temperature on color intensity. The color developed in the supernatant HCl hydrolysis of Carnoy fixed rat thymus cells when kept at 7°, 23°, 38°C. after addition of Feulgen's reagent was greatest at 7°C. (values 7° — 104, 28° — 82, 38° — 50).

The color developed in sections of rat duodenum after 24 minutes hydrolysis at 60° in 1 N HCl was also greater at 7° than at 23° or 38°C. The color at 38° was slightly diffuse; at 63° staining was faint and diffuse. The clearest and sharpest confinement of color to the nuclei occurred in the 7° color developed section.

Influence of bisulfite content of Feulgen reagent on color intensity. The sulfur dioxide content of the Feulgen reagent is not constant unless escape of the gas is prevented. The staining quality of the reagent deteriorates rapidly when the reagent container is kept open at room temperature. In order to test the influence of the SO₂ content on the color-producing potentialities of the reagent a solution of desoxyribo-

nucleic acid was hydrolyzed for 100 minutes at 60°C. To 1 cm³ aliquots of the hydrolyzate 1 cm³ of Feulgen's reagent was added. Increasing amounts of dry potassium metabisulfite were added to the tubes. Color was allowed to develop for 70 minutes at 25°C.; the color intensity was then read (table 4). It may be seen that color intensity increased greatly as the concentration of bisulfite was increased.

Bubbling oxygen through hydrolyzed desoxyribonucleic acid to which Feulgen's reagent had been added prevented color development. That non-color development was not caused by

TABLE 4

Influence of SO₂ on the intensity of Feulgen color with hydrolyzed desoxyribonucleic acid

K-META-BISULFITE ADDED	TOTAL BISULFITES PRESENT	COLORIMETRIC READINGS
<i>mg</i>	<i>mg</i>	
0	18.2	53
18	36.2	186
25	43.2	194
40	58.2	278
60	78.2	365
80	98.2	500
100	118.2	600

oxidation was shown when, by bubbling nitrogen through a similar solution, color did not develop. Color developed quickly in both solutions, however, when sulfur dioxide was bubbled through them. Apparently bubbling oxygen through the mixture effected a rapid removal of SO₂; without SO₂ staining failed.

Storage conditions for Feulgen's reagent

Feulgen's reagent does not retain its staining power well as ordinarily kept in the laboratory. In order to determine if the time of usefulness of samples of the reagent could be extended various storage conditions were studied. Samples were kept for two months under the following conditions: (1) in a partially filled container kept at room temperature; (2)

in completely filled containers, stopper sealed with paraffin, and kept at room temperature; (3) in a completely filled container, stopper sealed with paraffin, kept at 7°C.

Sections stained lightly and diffusely with the reagent that had been stored for two months at room temperature in a partially filled container; they stained well in the reagent that had been kept in a completely filled and sealed container at room temperature but best in the reagent that had been kept at 7°C. in a completely filled and sealed container.

DISCUSSION

Ultraviolet light photomicrographs (figs. 3-8) demonstrate clearly that hydrochloric acid hydrolysis, as performed in the Feulgen technic, quickly removes from the cytoplasm of Carnoy-fixed tissue cells the ultraviolet light absorbing constituents of ribonucleic acid. Carr ('45) suggested that hydrolysis destroys much of the cytoplasm of cells and that the localization of the stain in the Feulgen reaction might be because only a ghost of the cytoplasm is left after hydrolysis. The latter contention obviously is incorrect since the ribose even if it were present in the tissue after hydrolysis of the cytoplasmic ribonucleic acid would not recolorize the Feulgen reagent. The conclusion of Dodson ('46) that the efficiency of acid hydrolysis in destroying fresh cytoplasm does not extend to fixed tissue also is incorrect to the extent that ribonucleic acid is removed.

Ribonucleic acid is so abundant in the cytoplasm of the cells of the crypts of Lieberkühn of the rat intestine that nuclear detail is obscure in ultraviolet light photomicrographs (fig. 3). One to three minutes' hydrolysis removes sufficient ribonucleic acid to make nuclear detail clear. Such sections have the appearance of ribonuclease treated sections. This information might be of practical use in the cytological studies where large amounts of ribonucleic acid in cells obscure nuclear details under study.

Removal of the ultraviolet light absorbing constituents of desoxyribonucleic acid from nuclei by hydrolysis is much

slower than removal of ribonucleic acid from the cytoplasm. The duration of hydrolysis required to remove the purine and pyrimidine constituents from nuclei varied from 20 to 40 minutes in the several experiments whereas one to 6 minutes' hydrolysis removed the absorbing constituents of ribonucleic acid. Whether or not the longer time of hydrolysis is required to remove desoxyribonucleic acid because of its greater degree of polymerization or whether its linkage to protein is more stable than that of ribonucleic acid is not known.

The generally accepted explanation of the mechanism of the Feulgen reaction is that hydrochloric acid hydrolysis removes purines from the nucleic acid molecule, leaving exposed aldehyde groups of desoxyribose which combine with reduced fuchsin to produce a purple color. Hydrolysis, as carried out in the Feulgen technic, removes not only purines and pyrimidines but aldehydes as well since the hydrolyzate of fixed cell suspensions recolorizes the Feulgen reagent. Apparently the aldehydes in the hydrolyzate are not free but in combination in thymic acid molecules linked in chain formation. This assumption seems probable because the fragments of hydrolyzed desoxyribonucleic acid do not dialyze through cellophane and correspond to molecular weights from approximately 15,000 to 30,000; the average fragment would correspond to about 20 tetranucleotides. That the fragments are thymic acid multiples is probable because the comparatively mild Feulgen hydrolysis would remove purines but not pyrimidines; Kossell and Steudel as quoted by Levene ('31) found that mild hydrolysis which was sufficient to remove purines did not set free pyrimidines, heating with 20 to 40% sulfuric acid at about 150°C. being required to remove any pyrimidines. Purine bases were destroyed by this severe hydrolysis.

Whether depolymerization of nucleic acid precedes or follows splitting off of purines is not known.

Removal of ultraviolet light absorbing constituents progresses to completion when hydrolysis is continued sufficiently. The time of hydrolysis necessary for complete removal

varied somewhat (20–40 minutes) in different experiments. As hydrolysis was continued and increasing amounts of ultra-violet light absorbing constituents were removed the color developed in both cells and hydrolyzate increased to a maximum at approximately the same time and then declined. These facts are interpreted in the following manner: when purines are removed from the desoxyribonucleic acid molecule the resultant aldehyde groups recolorize the Fuchsin reagent. The aldehyde remains attached to the thymic acid. If the nucleic acid (or thymic acid) remained attached to protein in the cell no color would develop in the hydrolyzate. Since color does develop in the hydrolyzate, fragments of partially depolymerized desoxyribonucleic acid or polytetranucleotides of thymic acid must be detached from the cells. Since color in both phases increases with time of hydrolysis, splitting off of purines, depolymerization, and detachment of polytetranucleotides of thymic acid must proceed together. When the original nucleic acid is entirely removed from the cells color no longer develops in the cells. At this time ultraviolet absorbing constituents have been removed. Normally one would expect that at this time the maximum color would develop in the hydrolyzate but this is not so. As hydrolysis of nucleic acid solutions is continued the concentration of products capable of recolorizing Feulgen's reagent reaches maximum then declines. The products, apparently, are altered by continued hydrolysis so that they no longer recolorize the Fuchsin reagent. Aldehydes recolorize the reagent, the corresponding acids do not. For many years the sugar of desoxyribosyl-purines was thought to be a hexose because on hydrolysis levulinic acid was invariably isolated. Change of aldehyde groups to acid groups by long continued hydrolysis would account for decreased color formation as hydrolysis is continued.

Stacy, Deriaz, Teece and Wiggins ('46) suggest that the hydrolysis product which recolorizes the Feulgen reagent is ω -hydroxylevulinic aldehyde. Danielli ('47) contended that this aldehyde was so soluble and diffusible that it could not possibly be responsible for the reaction in tissue. The present

work indicates that the aldehydes removed are attached to phosphorus and that levulinic aldehyde is not formed during the Feulgen hydrolysis; the aldehydes that recolor Feulgen's reagent may be converted to acids with less intense hydrolysis than is required to break the sugar phosphate linkage to produce levulinic acid.

The contention of Stedman and Stedman ('43, '47), and others, that HCl hydrolysis of tissues removes nucleic acid cannot be denied. Their contention that the color formed by combination of aldehyde and Feulgen reagent in the tissue may diffuse and stain other structures in cells, and, in this way not indicate the true location of desoxyribonucleic acid, cannot be affirmed or denied on the basis of the present data. The color produced by hydrolyzed desoxyribonucleic acid and Feulgen reagents acts as a stain and stains cell structures intensely with little evidence of selectivity when sections are immersed in the already developed color solution. Conditions are somewhat different when hydrolyzed sections are placed in Feulgen's reagent; in this case the aldehyde groups that recolorize the reagent still may be adherent in their original location, those that hydrolysis detached having diffused from the tissue during hydrolysis.

The results of the experiments described in this paper support the contention that the Feulgen reagent is specific for desoxyribonucleic acid in that the color is formed by combination with products derived from the nucleic acid. It would seem, however, that exact quantitative work with the method would be difficult. As hydrolysis proceeds potential color-producing fragments are constantly being removed from the tissue. Intensity of color is influenced by changes in temperature, sulfite concentration and acid concentration. Various investigators have used from 6 to 15 minutes hydrolysis in the Feulgen technic. A longer time is usually necessary for development of the greatest intensity of color; the time may vary with tissues, and more so with various fixatives. The bond between nucleic acid and protein may be stronger in some cells than in others or may vary from time to time in

the same cell. Six minutes' hydrolysis removes less nucleic acid from sections than a longer time of hydrolysis; however, this comparatively short time of hydrolysis yields less potential color producing aldehyde groups than a longer hydrolysis. What constitutes the proper amount of hydrolysis is uncertain at present.

CONCLUSIONS

Hydrolysis of sections of tissue as carried out in the Feulgen technic was found to remove both ribonucleic acid and desoxyribonucleic acid from cells. Ribonucleic acid was removed rapidly, desoxyribonucleic acid more slowly.

Feulgen hydrolysis was found to depolymerize desoxyribonucleic acid, and remove purines; the unremoved polytetranucleotides of thymic acid which contain the aldehyde groups in part remain attached to the protein of the cells and in part become detached and diffuse into the hydrolyzate.

The Feulgen nucleal reaction was found to be specific for desoxyribonucleic acid in the sense that color is produced by combination of hydrolysis products of the nucleic acid with the Feulgen reagent. The technic is satisfactory for qualitative work but quantitatively there is loss of Feulgen reacting substance from the cell.

Feulgen's reagent may be kept for longer periods of time when containers are full and sealed and kept cold than when kept in partially filled unsealed containers.

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PLATE 1

EXPLANATION OF FIGURES

3 Section of unhydrolyzed tissue from rat duodenum. Photomicrograph taken at 2654 Å. $\times 425$.

4 Section hydrolyzed 1 minute in 1 N HCl at 60°C. Photomicrograph taken at 2654 Å. $\times 425$.

5 Section hydrolyzed 3 minutes in 1 N HCl at 60°C. Photomicrograph taken at 2654 Å. $\times 425$.

6 Section hydrolyzed 6 minutes in 1 N HCl at 60°C. Photomicrograph taken at 2654 Å. $\times 425$.

7 Section hydrolyzed 12 minutes in 1 N HCl at 60°C. Photomicrograph taken at 2654 Å. $\times 425$.

8 Section hydrolyzed 20 minutes in 1 N HCl at 60°C. Photomicrograph taken at 2654 Å. $\times 425$.

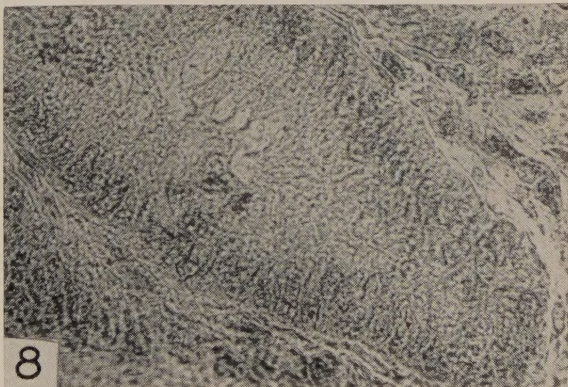
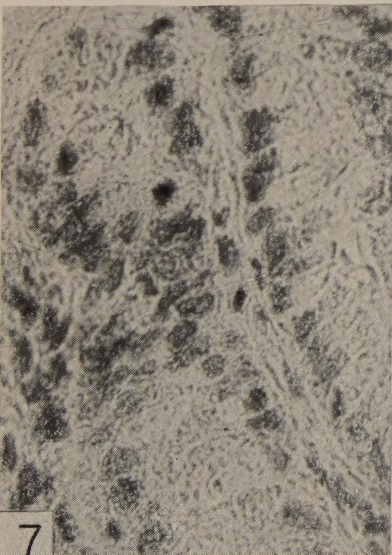
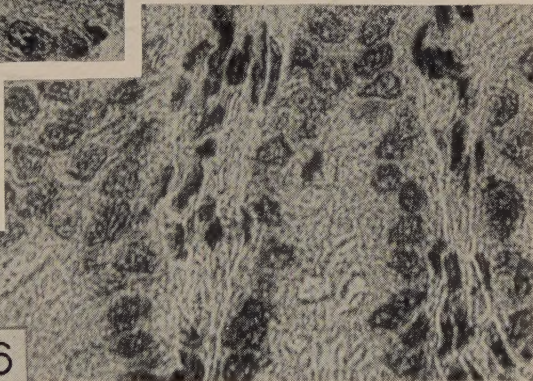
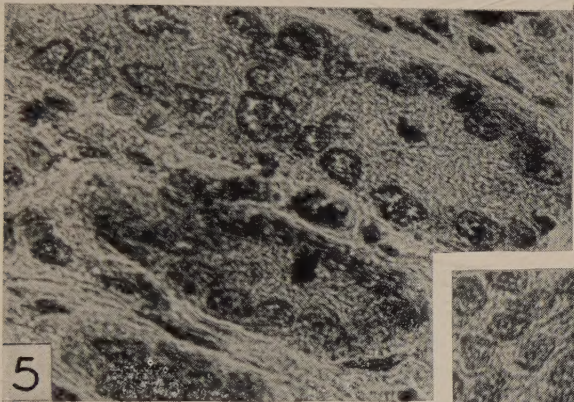
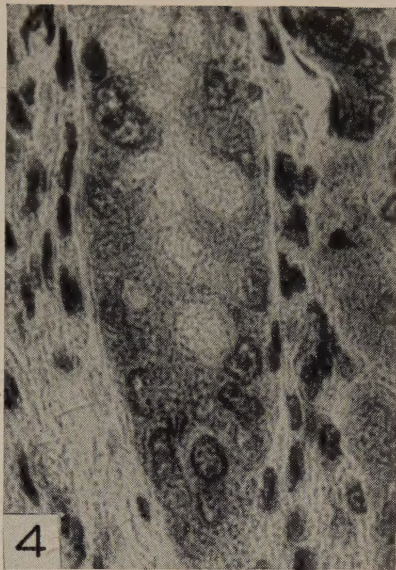
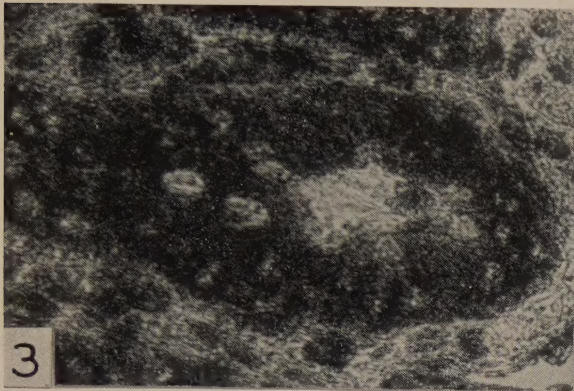


PLATE 2

EXPLANATION OF FIGURES

9 Ash of unhydrolyzed section of rat duodenum. Darkfield photomicrograph. $\times 200$.

10 Ash of section of rat duodenum hydrolyzed (1 N HCl, 60°C.) 1 minute. Darkfield photomicrograph. $\times 200$.

11 Ash of section of rat duodenum hydrolyzed (1 N HCl, 60°C.) 10 minutes. Darkfield photomicrograph. $\times 200$.

12 Ash of section of rat duodenum hydrolyzed (1 N HCl, 60°C.) 20 minutes. Darkfield photomicrograph. $\times 200$.

